



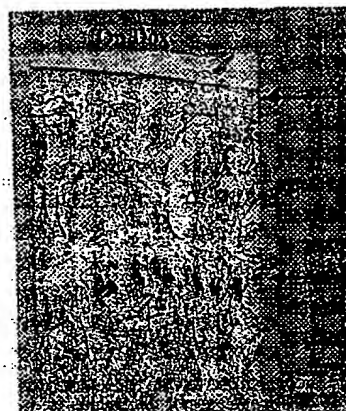
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(54) Title: **TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE**

(57) Abstract

The present invention provides animal model systems for cartilage-degenerative disease, which comprise transgenic animals which can express recombinant matrix-degrading enzymes (MDEs), particularly matrix metalloproteinases (MMPs), in a temporally and spatially regulated manner. The invention also provides methods for producing phenotypic indicators of cartilage-degenerative disease in a mammal and methods for determining the potential of a composition to counteract cartilage-degenerative disease. The invention also provides isolated nucleic acids encoding proMMP polypeptides that exhibit constitutive enzymatic activity and isolated proMMP polypeptides.

**A**

Articular Cartilage

Growth Plate

B
Articular Cartilage

Growth Plate



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TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE

5 Field of the Invention

The present invention pertains to transgenic mammals that express recombinant matrix-degrading enzymes in a temporally and spatially regulated manner. The invention further pertains to model systems incorporating such transgenic mammals for studying degenerative joint diseases, including systems for identifying therapeutic
10 agents and treatment regimens.

Background of the Invention

Degenerative diseases of cartilage, including joint and disc diseases such as osteoarthritis, rheumatoid arthritis, and osteochondrodysplasias, are widespread,
15 particularly in the elderly. Early symptoms common to these diseases include progressive loss of proteoglycans in the joint (as evidenced by loss of metachromasia); collagen degradation; fibrillation of the cartilage surface; and, ultimately, loss of cartilage (which is evidenced radiologically as joint space narrowing).

One of the primary targets affected by these diseases is type II collagen,
20 the major structural collagen found in articular cartilage. There is a balance between the production of type II collagen and catabolic enzymes that degrade type II collagen during normal remodeling of cartilage and bone. Pathological conditions such as, e.g., degenerative joint diseases, may result when this balance is disrupted.

Among the enzymes that degrade extracellular matrix components are
25 matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes, and aggrecanase (Table 1).

Table 1

Matrix-Degrading Enzymes							
	SUBSTRATES						
	Collagen	Gelatin	Proteoglycan	Fibronectin	Laminin	Elastin	Other
5	I. Metalloproteinases						
	Collagenases						
	MMP-1 (intestinal collagenase)	I, II, III, VII, X	✓				
10	MMP-8 (neutrophil collagenase)	I, II, III					
	MMP-13 (collagenase 3)	I, II, III	✓				
	Gelatinases						
15	MMP-2 (gelatinase A)	IV, V, VII, XI	✓	✓	✓	✓	
	MMP-9 (gelatinase B)	IV, V	✓	✓			
	Stromelysins						
20	MMP-3 (stromelysin 1)		✓	✓	✓		activates MMP zymogens
	MMP-7 (matrilysin)	IV	✓	✓	✓	✓	
	MMP-10 (stromelysin 2)	IV, V, IX		✓	✓		activates MMP zymogens
25	MMP-11 (stromelysin 3)	IV		✓	✓		activates serpins
	Other						
	MMP-12 (metalloelastase)					✓	
30	MMP-14		✓				proMMP- 2, proMMP- 13
	MMP-15						
	MMP-16						proMMP- 2
	MMP-17						
35	II. Aggrecanase		✓				

MMPs are synthesized in articulating joints by chondrocytes, which, in mature articular cartilage, are terminally differentiated cells that maintain the cartilage-

specific matrix phenotype. Overexpression of MMPs relative to endogenous MMP inhibitors, as occurs in degenerative joint diseases, may result in cartilage degradation. For example, Type II collagen is a substrate for MMP-13 and MMP-1 (Knauper et al., *J. Biol. Chem.* 271:1544, 1996) and both MMP-1 and MMP-13 proteins can be detected
5 immunohistochemically in human osteoarthritic tissues. In some cases, MMP-13 and its cleavage products are found at higher levels than MMP-1 (Billinghurst et al., *J. Clin. Invest.* 99:1534, 1997). Thus, MMP-13 may play an important role in cartilage degradation associated with osteoarthritis and other degenerative joint diseases (Mitchell et al., *J. Clin. Invest.* 97:761, 1996).

10 Animal models for osteoarthritis-related syndromes have been described in guinea pigs (Watson et al., *Arth. Rheum.* 39:1327, 1996) and in the inbred STR/ORT strain of mice (Das-Gupta et al., *Int. J. Exp. Path.* 74:627, 1993). In guinea pigs, spontaneous osteoarthritis has a long course of development (six months or more), and only certain
15 sublines of STR/ORT mice consistently develop degenerative joint disease. Thus, the duration and/or variability of these models renders them less applicable to drug discovery studies.

Other osteoarthritis-related models include surgically-induced joint destabilization, e.g., anterior cruciate ligament transection and/or partial meniscectomy in rabbits and dogs, which stimulates cartilage degradation (Hulth et al., *Acta Orthop. Scand.*
20 41:522, 1970). Another model employs injection of bacterial collagenase into the joints of an animal to induce a biochemical ligament transection (Van der Kraan et al., *J. Exp. Pathol.* 71:19, 1990). Because (i) surgical or other manipulation of individual animals is required; (ii) the animals are large and expensive; and/or (iii) the course of disease is not consistent, these models cannot easily be used in large-scale studies, including drug
25 screening.

Transgenic animal models, in principle, can provide the opportunity for a reproducible animal model system for degenerative joint diseases. However, previous attempts to engineer transgenic animals expressing MMPs such as MMP-1 and stromelysin have not resulted in an observable joint degeneration phenotype in the
30 transgenic animals. This could be due to embryonic lethality caused by constitutive

expression of these enzymes. Witty et al., *Mol. Biol. Cell* **6**:1287, 1995, have created transgenic animals that constitutively express MMP-1 and stromelysin in mammary tissue, but these animals do not exhibit symptoms of osteoarthritis. D'Armiento et al., *Cell* **71**:955, 1992, disclose transgenic mice that express human interstitial collagenase in the lung. Liu et al., *J. Cell Biol.* **130**:227, 1995, disclose transgenic animals that overexpress mutated type II collagen, resulting in connective tissue defects but not osteoarthritis. None of these transgenic animal systems provides a useful animal model for osteoarthritis (Khokha et al., *Cancer and Metastasis Rev.* **14**:97, 1995; Shapiro, *Matrix Biol.* **15**:527, 1997).

Thus, there is a need in the art for animal model systems that mimic human degenerative joint diseases such as, e.g., osteoarthritis, rheumatoid arthritis, and chondrodysplasias. Transgenic animals containing regulatable heterologous genes whose expression results in cartilage degeneration are particularly advantageous in providing reproducible experimental control over the timing and the level of expression of the transgenes and, thereby, over the pathological syndrome itself. Such animals can be used to determine what level of expression of the transgene is required to cause disease and, importantly, can be used for drug discovery and optimization of treatment regimens. In particular, such transgenic animals can be used to further define the role of matrix-degrading enzymes in cartilage degradation and as an *in vivo* screen to identify compounds that modulate these enzymes or compounds that inhibit the progression of degenerative joint diseases.

Summary of the Invention

The present invention provides transgenic non-human animals or the progeny thereof whose somatic and germline cells contain, in stably integrated form, one or more heterologous or recombinant genes encoding polypeptides comprising enzymatically active matrix-degrading enzymes (MDEs), preferably MMPs. MMPs for use in the invention comprise one or more of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-15, MMP-16, and MMP-17; preferably one or more of MMP-1, MMP-3, MMP-8, and MMP-13; and most

preferably one or more of MMP-1 and MMP-13; and include enzymatically active variants, fragments, and combinations of these polypeptides. Other matrix-degrading enzymes can also be used, including, e.g., aggrecanase. The MDEs may be derived from any species, preferably human. In preferred embodiments, the recombinant MDE-
5 encoding genes are selectively expressed in articular chondrocytes of the transgenic animal and expression results in pathological symptoms characteristic of degenerative joint disease.

In one aspect, the invention provides a transgenic animal or the progeny thereof whose somatic and germline cells contain a stably integrated first recombinant
10 gene encoding an MDE or an enzymatically active derivative or variant thereof, preferably a constitutively active proMMP-13 variant (designated MMP-13*) comprising the sequence depicted in SEQ ID NO: 4. Preferably, the first recombinant gene is under the control of a first regulatable promoter; most preferably, the first regulatable promoter comprises a tetO7 sequence, such as, e.g., the promoter depicted in SEQ ID NO: 5. The
15 transgenic animal may further comprise a second recombinant gene encoding a polypeptide that regulates the first regulatable promoter and is preferably a tTA polypeptide. In these embodiments, the second recombinant gene is under the control of a second regulatable promoter, preferably one that comprises sequences derived from a joint-specific promoter, and most preferably a type II collagen promoter, such as, e.g., the
20 promoter depicted in SEQ ID NO: 6. Selective expression of the second recombinant gene in joint tissues thus results in regulated joint-specific expression of the recombinant MDE.

In another aspect, the invention provides isolated nucleic acids encoding enzymatically active MMP variants, preferably human proMMP-13 variants, and most preferably MMP-13*. The invention also encompasses recombinant cloning vectors
25 comprising these nucleic acids; cells comprising the vectors; methods for producing MMP-13-derived polypeptides comprising culturing the cells under conditions appropriate for MMP-13 expression; and isolated MMP-13-derived polypeptides.

In yet another aspect, the invention provides methods for producing phenotypic changes characteristic of cartilage-degenerative disease in a mammal, which
30 comprise exposing the transgenic animals of the invention to conditions that result in

expression of the MDEs encoded by the transgenes. In a preferred embodiment, a transgenic animal comprising a first recombinant gene encoding MMP-13* operably linked to a tet07 promoter and a second recombinant gene encoding a tTA protein operably linked to a type II collagen promoter is maintained in the presence of tetracycline or a tetracycline analogue. When it is desired to induce expression of MMP-13*,
5 tetracycline or the tetracycline analogue is withdrawn, MMP-13* is selectively expressed in joint tissues, and phenotypic changes characteristic of cartilage-degenerative disease result.

In yet another aspect, the invention provides methods for determining the
10 potential of a composition to counteract cartilage-degenerative disease. The methods are carried out by administering a known dose of the composition to the transgenic animals of the invention, either before or after phenotypic indicators of cartilage-degenerative disease have developed; monitoring the indicators for a predetermined time following
administration of the composition; and comparing the extent of the indicators in the animal
15 to which the composition was administered relative to a control transgenic animal that had not been exposed to the composition. Any difference in (i) the nature or extent of phenotypic indicators of cartilage-degenerative disease, (ii) the time required for the indicators to develop, or (iii) the need for other ameliorative treatments indicates the potential of the composition to counteract cartilage-degenerative disease.

20

Brief Description of the Drawings

Figure 1A and 1B. (A) Schematic illustration of the structure of human MMP-13 (collagenase-3). The first box (corresponding to the extreme aminoterminal) represents the pre domain (signal peptide) that targets nascent proMMP-13 for secretion.
25 The second box represents the pro domain, which is involved in maintaining the latency of the enzyme. A conserved sequence (SEQ ID NO:1) within the pro domain that is important for maintaining enzyme latency is shown. The third box represents the 170-amino acid catalytic domain, which contains a conserved region (shown; SEQ ID NO:2) that is important for catalytic activity. The fourth box represents the 200-amino acid
30 carboxyterminal domain. (B) Illustration of the nucleic acid sequence (SEQ ID NO:3)

encoding a constitutively active variant of human pro MMP-13, designated MMP-13*, and the amino acid sequence of MMP-13* (SEQ ID NO:4). The residues that are mutated relative to wild-type MMP-13, which are depicted in larger type, are GTC at nucleotide positions 299-301.

5 **Figures 2A and 2B.** Binary strategy used to obtain chondrocyte specific, doxycycline (DOX) regulated MMP13* expression. (A) The first construct illustrates the rat type II collagen promoter driving expression of the tetracycline repressor - VP16 activator fusion protein (TA), followed by an SV40 splice and polyadenylation signal (this construct is referred to as CPE - TA). (B) The second construct illustrates the Tet07
10 promoter (Gossen and Bujard, *Proc. Natl. Acad. Sci. USA*, **89**:5547, 1992), driving expression of a constitutively active human MMP13 protein, followed by an SV40 splice and polyadenylation signal (construct referred to as Tet07 - MMP13*). These two independent transgene constructs were co-microinjected into fertilized mouse embryos to generate a double transgenic harboring both genes. In the presence of DOX, transgene
15 expression is off; when DOX is removed, transgene expression is turned on. An arrow denotes the transcription start site, while the asterisk indicates a constitutively active mutation in the MMP13 transgene.

Figure 3A and 3B. The type II collagen gene promoter directs expression to the joints of tester transgenic mice expressing β -galactosidase under the control of the
20 rat type II collagen promoter. (A) Diagram of the CPE -lacZ construct. The rat type II collagen promoter (first box) drives expression of the β -galactosidase (lacZ) gene (second box), which is followed by a β -globin splice and polyadenylation signal (final boxes joined by splice symbol). (B) Whole mount staining for β -galactosidase activity on E16 embryos. The embryo on the left shows the staining (arrows) of the transgenic compared
25 to a wild-type embryo. (C) Higher magnification of a transgenic elbow and front paw; β -gal staining is visible (arrows). Figures 3B and 3C more dramatically illustrate the invention when rendered in color, although black and white rendering is sufficient for understanding the invention.

Figure 4A and 4B. Expression profile of the TA and MMP13 RNA by
30 RT-PCR. (A) Amplification of TA cDNA from total RNA. (B) Amplification of

MMP13* cDNA from total RNA. Lane 1: $\times 164$ Hae III MW markers; Lane 2: PCR amplification of transgenic (line 6) genomic DNA; Lane 3: PCR amplification of non-transgenic genomic DNA; Lane 4: a wild-type mouse maintained on DOX; Lane 5: a wild-type mouse off DOX; Lanes 6-7: transgenic mice (~four months) maintained on DOX; Lanes 8-9: transgenic mice (~four months) removed from DOX at birth. The arrows 1a and 1b indicate a 648 bp MMP13* specific fragment and a 859 bp specific fragment, respectively. Each reaction was run using c-fos primers as an internal control, spliced mRNA yielding 187 bp (arrow 3) and unspliced mRNA 303 bp (arrow 2). No bands were detected in corresponding lanes containing RNA for PCR that was not treated with RT (data not shown).

Figure 5A and 5B. Photographic illustrations of immunohistochemical localization of type II collagen cleavage products in the growth plate and articular cartilage of transgenic mice expressing the transgenes shown in Figure 2. The tissues were stained with an antibody that recognizes cleavage products of type II collagen. (A) Tissue derived from a mouse that had been maintained on doxycycline to repress MMP-13* expression. (B) Tissue derived from a mouse that had been withdrawn from doxycycline, allowing expression of MMP-13*, for 30 days at 3 months of age. Figures 5A and B more dramatically illustrate the invention when rendered in color, although black and white rendering is sufficient for understanding the invention.

Figure 6A, 6B, and 6C. A color photographic illustration of Safranin O staining of the articular cartilage and growth plate of the patella of double transgenic mice. (A) Tissue derived from a mouse maintained on doxycycline. (B) Tissue derived from a mouse 7 days after withdrawal from doxycycline. (C) Tissue derived from a mouse 14 days after withdrawal from doxycycline. Figures 6A-C more dramatically illustrate the invention when rendered in color, although black and white rendering is sufficient for understanding the invention.

Figures 7A, 7B, 7C, and 7D. Longitudinal section through the hind knee joints (A and B) and synovium (C and D). (A and C) Age match littermate control and (B and D) line 6 removed from DOX. Abbreviations: L, lesion; AC, articular cartilage; AG, angiogenesis, IH, infiltration hyperplasia; BM, bone marrow; and, PL, patella

ligament. The location of the femur and patella are labelled on the figure. Figures 7A-D more dramatically illustrate the invention when rendered in color, although black and white rendering is sufficient for understanding the invention.

5 Detailed Description of the Invention

The present invention is based on the discovery regulated expression of matrix-degrading enzymes in cartilage in transgenic mice results in characteristic phenotypic changes associated with matrix degenerative diseases of the joints and intervertebral discs. The animal models of the invention provide novel model systems for
10 matrix degenerative disease syndromes which can be used for detailed characterization of human joint and intervertebral disc pathologies as well as for drug discovery and optimization of treatment regimens.

A transgenic animal according to the invention is an animal having cells that contain a transgene which was introduced into the animal or an ancestor of the animal
15 at a prenatal (embryonic) stage. A transgenic animal can be created, for example, by introducing the gene of interest into the male pronucleus of a fertilized oocyte by, e.g., microinjection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The gene of interest may include appropriate promoter sequences, as well as intronic sequences and polyadenylation signal sequences. Methods for producing
20 transgenic animals are disclosed in, e.g., U.S. Patent Nos. 4,736,866 and 4,870,009 and Hogan et al., *A Laboratory Manual*, Cold Spring Harbor Laboratory, 1986. A transgenic founder animal can be used to breed additional animals carrying the transgene. A transgenic animal carrying one transgene can also be bred to another transgenic animal carrying a second transgene to create a "double transgenic" animal carrying two
25 transgenes. Alternatively, two transgenes can be co-microinjected to produce a double transgenic animal. Animals carrying more than two transgenes are also possible. Furthermore, heterozygous transgenic animals, i.e., animals carrying one copy of a transgene, can be bred to a second animal heterozygous for the same transgene to produce homozygous animals carrying two copies of the transgene.

30 The present invention encompasses transgenic animals, preferably

mammals, which express MDEs, particularly MMPs, and most particularly those MMPs having collagenase activity, from a recombinant gene. MDEs for use in the invention include without limitation MMPs and aggrecanase. Useful MMPs include without limitation the collagenases designated MMP-1, MMP-8 and MMP-13; the stromelysins designated MMP-3, MMP-10, and MMP-11; the gelatinases designated MMP-2 and MMP-9; the metalloelastase designated MMP-12; and membrane-type MMPs designated MMP-14, MMP-15, MMP-16, and MMP-17 (Matrisian, *BioEssays*, 14:455, 1992).

Matrix-degrading activity as used herein refers to the proteolytic degradation of matrix components, including, e.g., collagen, particularly type II collagen and most particularly the triple helical form of type II collagen. Any polypeptide exhibiting matrix-degrading activity may be used in practicing the invention, including enzymatically active fragments of the above-described enzymes. Preferably, MMP-13 enzymatic activity is expressed. MMP-13 enzymatic activity as used herein refers to the proteolytic degradation of type II collagen. Any MMP-13 polypeptide or fragment or derivative thereof that exhibits MMP-13 enzymatic activity may be used. The enzymes may be derived from any animal species, including without limitation human, mouse, rat, rabbit, pig, cow, or non-human primate, or combinations thereof. Preferably, the MMP-13 or derivative thereof is of human origin.

Normally, MMPs are synthesized as precursors (i.e., zymogens or proenzymes) whose enzymatic activity is latent; proteolytic removal of the pro region after secretion produces the enzymatically active protein. In preferred embodiments of the invention, the need for proteolytic processing is circumvented by the use of enzyme or proenzyme variants that are enzymatically active even when uncleaved. Such variants can be produced using conventional techniques for site-directed or random mutagenesis coupled with analysis of collagenase enzymatic activity (see below). In this manner, modifications (including, e.g., insertions, deletions, and substitutions), may be introduced into a proenzyme sequence, particularly within the pro region or near the pro region cleavage site, to produce a constitutively active polypeptide which does not require proteolytic processing for activation. Alternatively, the pro region may be deleted entirely. Furthermore, recombinant genes may be used in which the sequence encoding

the native signal peptide is replaced by a heterologous sequence that functions as a signal peptide, i.e., promotes secretion. The use of genes encoding any such modified MMP polypeptides is encompassed by the invention.

Preferably, a constitutively active MMP-13 variant is used in practicing the invention. Most preferably, the MMP-13 variant comprises a sequence containing a mutation in the sequence encoding the PRCGVPDV region, SEQ ID NO:7, specifically a substitution of Pro⁹⁹ to Val; the sequence of this polypeptide is depicted in SEQ ID NO: 4 and this polypeptide is designated MMP-13*. In another embodiment, the constitutively active MMP-13 variant comprises a substitution of Val⁹⁸ to Gly.

The transgenic animals of the invention preferably express MMP activity in a regulated manner. Regulated expression as used herein refers to temporal and/or spatial control. Temporal control refers to the ability to repress expression of MMP activity until a predetermined time in the development of the transgenic animal, after which MMP expression may be activated and maintained for as long as desired. Preferably, MMP expression is repressed throughout embryonic development and activated in the adult animal. Spatial control refers to the ability to selectively express MMP activity in particular tissues. Preferably, MMP activity is selectively expressed in joint tissues, most preferably in articular chondrocytes.

Temporal control of MMP expression is achieved by use of one or more polypeptides comprising a transcriptional repressor, a transcriptional activator or enhancer, or combinations thereof, in conjunction with a promoter responsive to the transcriptional repressor/activator used to which the MMP-encoding sequence is operably linked. In one set of embodiments, temporal control of MMP expression is achieved by (i) expression in the transgenic animal of a repressor polypeptide operably linked to a polypeptide that directly or indirectly activates transcription in eucaryotic cells, creating a repressor-activator fusion polypeptide; and (ii) the coupled use of a target promoter operably linked to an MMP-encoding sequence whose transcriptional activity is responsive to the repressor-activator fusion polypeptide. Typically, nucleotide sequences encoding the repressor polypeptide are ligated in-frame to sequences encoding the transcriptional activator polypeptide to create a chimeric gene encoding a fusion protein.

Useful repressor polypeptides include without limitation polypeptides comprising sequences derived from bacterial repressors, including without limitation tetracycline repressor, LacR repressor, KRAB domain, and lambda repressor (cro and cI), as well as eukaryotic repressors, including without limitation those involved in amino acid or sugar synthesis. Useful direct transcriptional activator polypeptides include without limitation herpes simplex virus protein 16 (VP16); yeast GAL14; yeast STAT; steroid receptors such as, e.g., progesterone receptor and estrogen receptor; and constitutive activators such as, e.g., c-fos, c-jun, and SP-1. Alternatively, the repressor polypeptide may be linked to a polypeptide that indirectly activates transcription by recruiting a transcriptional activator to interact with the repressor-activator fusion protein; such indirect activator polypeptides include without limitation TATA Box Binding Protein (TBP) and basic transcription factors, including, e.g., basic transcription factor D.

According to the invention, each repressor-activator fusion protein is used in conjunction with a target promoter that is responsive to the particular fusion protein and that regulates transcription of an MDE-encoding sequence. Typically, the promoter comprises at least one operator sequence responsive to the repressor component of the repressor-activator fusion polypeptide, which is operably linked to at least a minimal promoter that supports transcription in eucaryotic cells. Examples of suitable repressor-responsive operator sequences include without limitation sequences derived from the tetracycline resistance operon encoded in Tn10 in *E. coli*, the lambda repressor operon, and the yeast GAL repressor operon. Examples of suitable eucaryotic promoters from which minimal promoters may be derived include without limitation the cytomegalovirus (CMV) IE promoter, PtK-1 (thymidine kinase) promoter, HSP (heat shock protein) promoter, and any eukaryotic promoter containing a TATA box. Minimal promoter sequences may be derived from these promoters by (i) creating deletion mutants using conventional methods and (ii) testing the ability of the resulting sequences to activate transcription in a cell line. U.S. Patent No. 5,650,298 discloses a repressor-activator fusion protein comprised of sequences derived from the tetracycline repressor fused to VP16 sequences, which is designated tTA, and a tTA-responsive promoter, designated tet07, which comprises a Tn10-derived sequence linked to a portion of the CMV IE

promoter.

Alternatively, temporal control is achieved by (i) expression in the transgenic animal of a heterologous or recombinant transcriptional activator polypeptide or polypeptides and (ii) the coupled use of a target promoter operably linked to an MMP-
5 encoding sequence whose transcriptional activity is responsive to the heterologous or recombinant transcriptional activator. Useful transcriptional activators include without limitation a modified ecdysone receptor, in which a VP16 transactivation domain linked to the aminoterminal transactivation domain of the glucocorticoid receptor is fused to the
10 ligand-binding domain and carboxyterminal sequence of the ecdysone receptor (No et al., *Proc. Natl. Acad. Sci. USA* 93:3346, 1996); a chimeric protein, designated pGL-VP, comprising VP16 activator sequences, GAL4 activation sequences, and a mutated human progesterone receptor ligand-binding domain (Wang et al., *Proc. Natl. Acad. Sci. USA* 91:8180, 1994; Wang et al., *Gene Therapy* 4:432, 1997); and chimeric proteins comprising transcriptional activators fused to estrogen (or other steroid) binding domains (Mattioni et
15 al., *Meth. Cell Biol.* 43:335, 1994). The ecdysone receptor system utilizes retinoid X receptor (RXR) to form heterodimers with the chimeric receptor, and responds to ecdysone, muristerone (an ecdysone analogue) or dexamethasone. The pGL-VP system is responsive to mifepristone (RU486). Chimeric receptors containing an estrogen binding domain respond to hydroxytamoxifen (an estrogen analogue).

20 Spatial control of MDE expression is achieved by the use of transcriptional promoters that direct transcription selectively in joint tissues. Joint-specific expression as used herein refers to expression that is greater in joints than in other cells; typically, the level of expression in non-joint tissues is less than 10% of the level of expression in joints. Preferably, expression in non-joint tissues is undetectable. Useful promoter sequences that
25 confer joint-specific expression on a sequence to which they are operably linked include without limitation sequences derived from the collagen type II promoter. It will be understood that a joint-specific promoter according to the invention may comprise one or more copies of particular sequences or sub-sequences, and these sequences may be in direct or inverted orientation relative to each other and relative to the sequence whose
30 expression is regulated by the promoter.

Coordinated spatial and temporal control of MDE expression is preferably achieved by (i) placing expression of the repressor-activator fusion polypeptide or the transcriptional activator polypeptide under the control of a joint-specific promoter; (ii) placing the expression of the MDE or a derivative thereof under the control of a promoter responsive to the repressor-activator fusion polypeptide or the transcriptional activator polypeptide; and (iii) maintaining the transgenic animal during fetal development and early life under conditions in which MDE expression is repressed.

The method by which transgenic animals are maintained during fetal and early post-natal development so that MDE expression is repressed will depend on the particular transgenes being expressed. When a repressor-activator fusion polypeptide is used, repression is achieved by providing the animal with an agent that binds to the repressor-activator fusion protein and results in repression of transcription of the target MDE gene. In animals comprising a transgene encoding a repressor-activator fusion polypeptide containing tet repressor sequences, repression is achieved by providing tetracycline or a tetracycline analogue in the food or drinking water of the mother and, following birth, of the progeny. Tetracycline or an analogue may also be provided using surgically implanted subcutaneous time-release pellets (Innovative Research of America, Inc., Sarasota FL). In this case, binding of tetracycline or a tetracycline analogue to the repressor-activator fusion protein prevents the fusion protein from binding to, and activating transcription of, the cognate promoter. Tetracycline analogues are compounds closely related to tetracycline which bind to the tet repressor with a K_a of at least about $10^6 M^{-1}$, preferably with an affinity of about $10^9 M^{-1}$ or greater. Useful tetracycline analogues include without limitation doxycycline, anhydrotetracycline, chlortetracycline, epioxytetracycline, and the like. The dosage used is one that will result in substantial repression of MMP expression. Typically, tetracycline or a tetracycline analogue is administered in the animal's drinking water at a dosage of about 1 mg/ml. When it is desired that MMPs be expressed, the tetracycline or analogue thereof is withheld.

In other embodiments, repression is achieved by withholding from the animal an agent required for activity of the transcriptional activator polypeptide. For example, if the transcriptional activator is a modified ecdysone receptor, the animals are

maintained in the absence of ecdysone or an ecdysone analogue throughout fetal and early post-natal development. Ecdysone analogues are compounds closely related to ecdysone which bind to the modified ecdysone receptor with a K_a of at least about $10^6 M^{-1}$. Useful ecdysone analogues include without limitation muristerone A. When it is desired that

5 MDEs be expressed, the animals are given, e.g., ecdysone or muristerone A via intraperitoneal injections at dosages of between about 10 mg and about 20 mg/animal. Similarly, when pGL-VP is used, activation is achieved by providing mifepristone.

In a preferred embodiment of the invention, a transgenic animal is constructed whose somatic and germline cells contain in stably integrated form two

10 recombinant genes: (i) a first recombinant gene comprising a sequence encoding MMP-13*, wherein the sequence is operably linked to a tetO7 promoter; and (ii) a second recombinant gene encoding a tTA protein operatively linked to a collagen type II promoter. In this embodiment, animals are maintained in the presence of tetracycline or a tetracycline analogue throughout fetal and early post-natal development to repress the

15 gene. Afterwards, tetracycline or the tetracycline analogue is withdrawn, and MMP-13 enzymatic activity is selectively expressed in joint tissues.

Animal Models for Cartilage-Degenerative Diseases

The present invention provides animal model systems in which phenotypic

20 changes characteristic of cartilage-degenerative diseases, such as, e.g., joint or disc disease, are reproducibly exhibited. These diseases include without limitation osteoarthritis, rheumatoid arthritis, chondrodysplasias, and degenerative intervertebral disc diseases. The model systems of the invention exhibit one or more phenotypic indicators common to these diseases, which include without limitation loss of proteoglycan (as

25 indicated by, e.g., loss of Safranin O staining) and cleavage of type II collagen in the affected tissues. The systems encompass the transgenic animals described above, in which recombinant or heterologous MDEs, particularly MMPs, are expressed in cartilage at a predetermined time in the life of the transgenic animal. The timing of the appearance of cartilage-degenerative indicators is determined by activating MDE expression and

30 monitoring the effects on cartilage (see below). Preferably, one or more MDEs are

expressed after birth, most preferably after the animal has reached adulthood.

Expression of the transgenes is typically monitored by extracting mRNA from different tissues and subjecting the extracted mRNA to one or more of the following: (i) reverse transcriptase-polymerase chain reaction (RT-PCR), using primers homologous to the transgene; (ii) RNAase protection; and (iii) Northern blot analysis. Alternatively, in situ hybridization may used.

The physiological effects of MDE expression on articular cartilage are monitored in test animals by sacrificing the animals and subjecting paraffin-embedded decalcified cartilage to staining with (i) hematoxylin and eosin (using conventional techniques) followed by double staining with (ii) Safranin O and fast green (Peter et al., *J. Exp. Pathol.* 71:19, 1990). Alternatively, frozen sections may be obtained and stained with antibodies that are specific for cleavage fragments derived from type II collagen (Billingham et al., *J. Clin. Invest.* 99:1534, 1997). Typically, expression of the MMP transgene(s) for at least about 7 days results in detectable loss of proteoglycan and changes in growth plate morphology (see, e.g., Example 5 below). Animal models in which expression of MDEs, particularly MMPs, and most particularly an enzymatically active form of MMP-13, results in proteoglycan loss and/or cleavage of type II collagen are within the scope of the invention.

Other phenotypic indicators of cartilage-degenerative disease which can be monitored in transgenic animals produced according to the invention include without limitation gross observations of changes in joint function and histological evidence of (i) fibrillation and loss of articular cartilage and (ii) osteophyte formation.

Syndromes for which the transgenic animals of the invention provide useful models include without limitation any pathological condition that manifests a disturbance in the composition, morphology, and/or function of cartilage, including osteoarthritis; rheumatoid arthritis; degenerative intervertebral disc diseases; chondrodysplasias, including, e.g., Kniest dysplasia, achondrogenesis, and hypophosphatasia; and proteoglycan-mediated disorders, such as occur, e.g., in brachymorphic animals (Hall et al., *Cartilage: Molecular Aspects*, CRC Press, 1991, pp. 201-203).

In further embodiments of the invention, the transgenic animals can be

subjected to additional treatments to modulate the cartilage-degenerative indicators and/or to supplement the animals' disease phenotype with additional physiological effects such as, e.g., those associated with a particular disease. For example, the transgenic animals may be further treated with inflammatory mediators to augment collagen degradation and/or induce loss of proteoglycan (see, e.g., Example 6 below). Furthermore, the timing and extent of MDE induction, with or without additional treatments, can be adapted to replicate the symptomatology of a particular disease or syndrome.

Methods for Evaluating Drugs that Modulate Degenerative Diseases of Cartilage

10 The present invention encompasses methods for discovery and evaluation of drugs and therapies for their efficacy against degenerative diseases of cartilage, particularly degenerative joint diseases. In one embodiment of the invention, the transgenic animals of the invention are maintained under conditions in which expression of one or more MDEs results in one or more phenotypic indicators of cartilage-

15 degenerative disease. Once the symptoms have developed, the potential of a composition to counteract cartilage-degenerative disease can be evaluated by administering a known dose of the composition to the animal in which the symptoms have developed; monitoring the phenotypic indicators for a predetermined time following administration of the composition; and comparing the extent of the phenotypic indicators in the animal to which

20 the composition was administered relative to a control animal. Control animals comprise age- and sex-matched transgenic animals that are maintained under an identical regimen (i.e., express the transgenes) but which do not receive the composition. Any statistically significant difference in the extent or nature of the phenotypic indicators indicates the potential of the composition to counteract cartilage-degenerative disease. As used herein,

25 phenotypic indicators of cartilage-degenerative disease refer to proteoglycan loss, joint space narrowing, collagen degradation, and destruction of cartilage.

 In another embodiment of the invention, the potential of a composition to counteract degenerative diseases of cartilage, particularly degenerative joint disease, is evaluated by administering to a transgenic animal a known dose of the composition before

30 and/or simultaneous with the induction of MDE expression in the transgenic animal;

monitoring phenotypic indicators of cartilage-degenerative disease for a predetermined time following administration of the composition and MDE induction; and comparing the extent of the phenotypic indicators and/or disease in the animal to which the composition was administered relative to a control animal that had not been exposed to the composition. In this embodiment, any statistically significant difference in the extent or nature of the phenotypic indicators and/or disease, or any statistically significant delay in appearance of the phenotypic indicators or disease, indicates the potential of the composition to counteract cartilage-degenerative disease.

A further indication of the potential of a composition to counteract cartilage-degenerative disease is the ability of the composition to cause any reduction in the extent or duration of other treatments, including, e.g., the dosage and timing of administration of other therapeutic agents used to alleviate symptoms of the disease.

Compounds that may be tested for anti-cartilage-degenerative disease potential may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, synthetic compound libraries, and compounds resulting from directed rational drug design and synthesis. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., *TibTech* **14**:60, 1996).

Transgenic animals

Transgenic animals as used herein refers to animals into which one or more heterologous and/or recombinant genes have been introduced. The transgenes may be from a different species, or from the same species as the transgenic animal but are not

naturally found in the animal in the configuration and/or at the chromosomal locus conferred by the transgene. Transgenes may comprise foreign DNA sequences, i.e., sequences not normally found in the genome of the host animal. Alternatively or additionally, transgenes may comprise endogenous DNA sequences that have been rearranged or mutated *in vitro* in order to alter the normal *in vivo* pattern of expression of the gene, or to alter or eliminate the biological activity of an endogenous gene product encoded by the gene. Also encompassed by the invention are DNA fragments that are introduced into a pre-existing gene to, e.g., change patterns of expression or to provide additional means of regulating the expression of the gene (Watson et al., "The Introduction of Foreign Genes Into Mice," in *Recombinant DNA*, 2d Ed., W.H. Freeman & Co., New York, 1992, pp. 255-272; Gordon, J.W., *Intl. Rev. Cytol.* 115:171, 1989; Jaenisch, *Science* 240:1468, 1989; Rossant, *Neuron* 2:323, 1990).

The transgenic non-human animals of the invention are produced by introducing transgenes into the germline of the non-human animal. Embryonal target cells at various developmental stages are used to introduce the transgenes of the invention. Different methods are used depending on the stage of development of the embryonal target cell(s). Such methods include, but are not limited to, microinjection of zygotes, viral integration, and transformation of embryonic stem cells as described below.

1. Microinjection of zygotes is the preferred method for incorporating transgenes into animal genomes. A zygote, which is a fertilized ovum that has not undergone pronuclei fusion or subsequent cell division, is the preferred target cell for microinjection of transgenic DNA sequences. The murine male pronucleus reaches a size of approximately 20 micrometers in diameter, a feature which allows for the reproducible injection of 1-2 picoliters of a solution containing transgenic DNA sequences. The use of a zygote for introduction of transgenes has the advantage that, in most cases, the injected transgenic DNA sequences will be incorporated into the host animal's genome before the first cell division (Brinster et al., *Proc. Natl. Acad. Sci. USA* 82:4438, 1985). As a consequence, all cells of the resultant transgenic animals (founder animals) stably carry an incorporated transgene at a particular genetic locus, referred to as a transgenic allele. The transgenic allele demonstrates Mendelian inheritance, i.e., half of the offspring resulting

from the cross of a transgenic animal with a non-transgenic animal will inherit the transgenic allele, in accordance with Mendel's rules of random assortment.

2. Viral integration can also be used to introduce the transgenes of the invention into an animal. The developing embryos are cultured *in vitro* to the blastocyte developmental stage. The blastomeres may be infected with appropriate retroviruses (Jaenich, *Proc. Natl. Acad. Sci. USA* 73:1260). Infection of the blastomeres is enhanced by enzymatic removal of the zona pellucida. Transgenes are introduced via viral vectors which are typically replication-defective but which remain competent for integration of viral-associated DNA sequences, including transgenic DNA sequences linked to such viral sequences, into the host animal's genome. Transfection is easily and efficiently obtained by culture of blastomeres on a monolayer of cells producing the transgene-containing viral vector. Alternatively, infection may be performed using cells at a later developmental stage, such as blastocoeles. In any event, most transgenic founder animals produced by viral integration will be mosaics for the transgenic allele; that is, the transgene is incorporated into only a subset of all the cells that form the transgenic founder animals. Moreover, multiple viral integration events may occur in a single founder animal, generating multiple transgenic alleles which will segregate in future generations of offspring. Introduction of transgenes into germline cells by this method is possible but probably occurs at a low frequency. However, once a transgene has been introduced into germline cells by this method, offspring may be produced in which the transgenic allele is present in all of the animal's cells, i.e., in both somatic and germline cells.

3. Embryonal stem (ES) cells can also serve as target cells for introduction of the transgenes of the invention into animals. ES cells are obtained from pre-implantation embryos that are cultured *in vitro* (Evans et al., *Nature* 292:154, 1981). ES cells that have been transformed with a transgene can be combined with an animal blastocyst, after which the ES cells colonize the embryo and contribute to the germline of the resulting animal (which is a chimera, i.e., composed of cells derived from two or more animals). Again, once a transgene has been introduced into germline cells by this method, offspring may be produced in which the transgenic allele is present in all of the animal's cells, i.e., in both somatic and germline cells.

Although the initial introduction of a transgene is a Lamarckian (non--Mendelian) event, the transgenes of the invention may be stably integrated into germ line cells and transmitted to offspring of the transgenic animal as Mendelian loci. Other transgenic techniques result in mosaic transgenic animals, in which some cells carry the transgenes and other cells do not. In mosaic transgenic animals in which germ line cells do not carry the transgenes, transmission of the transgenes to offspring does not occur. Nevertheless, mosaic transgenic animals are capable of demonstrating phenotypes associated with the transgenes.

In practicing the invention, animals of the transgenic maintenance line are crossed with animals having a genetic background in which expression of the transgene results in symptoms of cartilage-degenerative disease. Offspring that have inherited the transgenes of the invention are distinguished from littermates that have not inherited transgenes by analysis of genetic material from the offspring for the presence of nucleic acid sequences derived from the transgenes of the invention. For example, biological fluids that contain polypeptides uniquely encoded by the transgenes of the invention may be immunoassayed for the presence of the polypeptides. A simpler and more reliable means of identifying transgenic offspring comprises obtaining a tissue sample from an extremity of an animal, such as, for example, a tail, and analyzing the sample for the presence of nucleic acid sequences corresponding to the DNA sequence of a unique portion or portions of the transgenes of the invention. The presence of such nucleic acid sequences may be determined by, e.g., hybridization ("Southern") analysis with DNA sequences corresponding to unique portions of the transgene, analysis of the products of PCR reactions using DNA sequences in a sample as substrates, oligonucleotides derived from the transgene's DNA sequence, and the like.

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Nucleic Acids, Vectors, Expression Systems, and Polypeptides

The present invention encompasses isolated nucleic acids encoding MDEs, particularly MMPs, and enzymatically active fragments derived therefrom, as well as constitutively active MMP variants and enzymatically active fragments derived therefrom.

The invention also encompasses complements of the above nucleic acids; vectors

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comprising the nucleic acids; cells comprising the vectors; and isolated polypeptides encoded by the nucleic acids.

Many techniques in molecular biology, microbiology, recombinant DNA, and protein biochemistry are used in practicing the present invention, such as those explained in, for example, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); and *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.).

"Nucleic acid" or "polynucleotide" as used herein refers to purine- and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. This includes single- and double-stranded molecules, such as, for example, DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases.

A "coding sequence" or a "protein-coding sequence" is a polynucleotide sequence capable of being transcribed into mRNA and/or capable of being translated into a polypeptide. The boundaries of the coding sequence are typically determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus.

A "complement" of a nucleic acid sequence as used herein refers to the "antisense" sequence that participates in Watson-Crick base-pairing with the original sequence.

An "isolated" nucleic acid or polypeptide as used herein refers to a component that is removed from its original environment (for example, its natural environment if it is naturally occurring). An isolated nucleic acid or polypeptide typically contains less than about 50%, preferably less than about 75%, and most preferably less than about 90%, of the cellular components with which it was originally associated.

A nucleic acid or polypeptide sequence that is "derived from" a designated

sequence refers to a sequence that corresponds to a region of the designated sequence. For nucleic acid sequences, this encompasses sequences that are homologous or complementary to the sequence, as well as "sequence-conservative variants" and "function-conservative variants." For polypeptide sequences, this encompasses "function-conservative variants." Sequence-conservative variants are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Function-conservative variants are those in which a given amino acid residue in a polypeptide has been changed without altering the overall conformation and function of the native polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic, hydrophobic, and the like). "Function-conservative" variants also include any polypeptides that have the ability to elicit antibodies specific to a designated polypeptide.

Nucleic acids comprising any of the sequences disclosed herein or subsequences thereof can be prepared by conventional methods. For example, DNA can be chemically synthesized using, e.g., the phosphoramidite solid support method of Matteucci *et al.*, 1981, *J. Am. Chem. Soc.* 103:3185, the method of Yoo *et al.*, 1989, *J. Biol. Chem.* 764:17078, or other well known methods. This can be performed by sequentially linking a series of oligonucleotide cassettes comprising pairs of synthetic oligonucleotides.

Due to the degeneracy of the genetic code, many different nucleotide sequences can encode polypeptides having the amino acid sequences defined herein or subsequences thereof. The codons can be selected for optimal expression in prokaryotic or eukaryotic systems. Such degenerate variants are also encompassed by this invention.

The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Nucleic acids may contain

- one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. PNAs are also encompassed by the term "nucleic acid". The nucleic acid may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.
- 10 The polypeptides of the invention may be expressed by using many known vectors, such as pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), or pRSET or pREP plasmids (Invitrogen, San Diego, CA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. The particular choice of vector/host is not critical to the practice of the invention.
- 15 Recombinant cloning vectors will often include one or more replication systems for cloning or expression; one or more markers for selection in the host, such as, for example, antibiotic resistance; and one or more expression cassettes. The inserted coding sequences may be synthesized by standard methods, isolated from natural sources, prepared as hybrids, or the like. Ligation of the coding sequences to transcriptional regulatory elements and/or to other amino acid coding sequences may be achieved by known methods. Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including electroporation, CaCl_2 mediated DNA uptake, fungal infection, microinjection, microprojectile, or other established methods.
- 20 Appropriate host cells include bacteria, archebacteria, fungi, yeast, plant, and animal cells, and especially mammalian cells. Of particular interest are *E. coli*, *S. aureus*, *B. subtilis*, *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Schizosaccharomyces pombe*, SF9 cells, C129 cells, 293 cells, *Neurospora*, CHO cells, COS cells, HeLa cells, and immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda,
- 30 adenovirus, cytomegalovirus, and the like. A large number of transcription initiation and

termination regulatory regions have been isolated and are effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, etc. are known in the art (Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley, 1997). Under appropriate
5 expression conditions, host cells can be used as a source of recombinantly produced peptides and polypeptides.

The MDEs of the present invention, including function-conservative variants, may be isolated from native or heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) into which the protein-coding
10 sequence has been introduced and expressed. Alternatively, these polypeptides may be produced in cell-free protein synthesis systems, which may additionally be supplemented with microsomal membranes to achieve glycosylation and signal peptide processing of procollagenases. Furthermore, the polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation, exclusive
15 solid phase synthesis, partial solid phase methods, fragment condensation, or classical solution synthesis.

Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and
20 countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the protein
25 or against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

The construction and analysis of MMP variants and derivatives that exhibit enzymatic activity, and preferably constitutive enzymatic activity, can be achieved by routine application of conventional methods. First, a nucleic acid encoding an MMP is
30 modified either by site-directed or random mutagenesis, or is used in a construction

scheme as one segment of a fusion gene. Preferably, the procedure results in a modification either contained within the sequence encoding the pro region or near the pro region cleavage site; this includes deleting the pro region entirely. Alternatively, sequences may be constructed that encode fusion proteins either between enzymatically active MMP domains and other polypeptides, or between different MMPs. The modified nucleic acid is then used to program synthesis of a variant MMP, either in a cell-free system, in intact cells (including permeabilized cells), or in a transgenic animal. Preferably, either a cell-free system or a cell culture system is used to express the MMP variant or derivative. The extent of pro region cleavage is assessed by metabolic labelling and resolution of the MMP product by SDS-PAGE. Finally, MMP enzymatic activity is measured using conventional assays, such as, by quantifying the cleavage of natural substrates or model peptides, as disclosed, e.g., in Weingarten et al., *Biochem.* **24**:6730, 1985; Woessner et al., *J. Biol. Chem.*, **263**:16918, 1988, and Knight et al., *FEBS Letts.*, **296**:263, 1992. In this manner, a large number of MMP variants and derivatives, including, e.g., function-conservative variants of MMP-13*, can be created routinely and assayed for MMP enzymatic activity.

Description of the Preferred Embodiments

The following examples are intended to illustrate the present invention without limitation.

Example 1: Construction of a Gene Encoding a Modified, Constitutively Active ProMMP-13

The following experiments were performed to create a gene encoding a procollagenase derived from MMP-13 that is enzymatically active in the absence of pro region cleavage. The sequence of this proMMP-13 variant, designated MMP13*, is shown in SEQ ID NO:4.

Site-directed mutagenesis to modify MMP-13 cDNA

A cDNA fragment encoding proMMP was obtained by digesting plasmid pNot3A (Freije et al., *J. Biol. Chem.* **269**:16766, 1994; GENBANK accession number

X75308) with XbaI and HindIII and purifying the resulting ~1515 bp fragment. This fragment was subcloned into the Tet-resistant/Amp-sensitive pAlter plasmid (Promega, Madison, WI) that had been digested with XbaI and HindIII.

Site-directed mutagenesis was performed using the Altered Sites II in vitro Mutagenesis System (Promega, Madison, WI). Briefly, phagemid single-stranded DNA was purified from cultures containing the helper phage R408 (Promega). In addition to the Amp repair - Tet knock-out conversion oligos (Promega), an oligonucleotide having the sequence 5'-AAGCCAAGATGCGGGGTGTCGATGTGGGTGAATACAAT-3', SEQ ID NO:8, was phosphorylated and annealed to the single-stranded DNA, followed by mutant strand synthesis. The reaction mixture was then used to transform the repair-minus *E. coli* strain ES1301 *mutS*, and the culture was grown in ampicillin selective media. Plasmid DNA was isolated from isolated clones and transformed into JM109 cells, which were then plated on LB plates containing 120 µg/ml ampicillin.

The above procedure resulted in a proline-to-valine substitution at amino acid 99. The modified proMMP was designated MMP13* (SEQ ID NO:4).

Using a similar technique, site-directed mutagenesis was also used to introduce a valine to glycine mutation at amino acid 98. A mutagenic oligonucleotide having the sequence 5'-GAAAAAGCCAAGATGCGGGGGTCCTGATGTGGGTGAATAC-5', SEQ ID NO:9 was used as described above. This procedure resulted in a valine-to-glycine substitution at amino acid 98.

After confirmation of the above mutations by direct sequencing, cDNA encoding MMP13* cDNA was excised from the pAlter-MMP13* vector by digestion with EcoRI and HindIII.

25

Example 2: Determination of the Enzymatic Activity of MMP-13*

Materials and Methods

cDNAs encoding both mutant forms of MMP13 and wild-type MMP-13 were subcloned into a BS(SK-) vector (Stratagene) containing the CMV promoter (Xho I - Eco RI) and the SV40 splice poly (A)_n (Xba I - Nco I). Duplicate cultures of Hela cells (10 cm

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dishes) were transfected with 50 µg of these plasmids using the CaPO₄ precipitation method (Promega). Five hours later, cells were subjected to a 1-minute glycerol shock using a solution containing an equal volume of 2 X HBS + 30 % glycerol. This procedure is described in the Profection Mammalian Transfection Systems technical manual

5 (Promega).

Twenty-four hours following transfection, the culture medium (D-MEM containing 10 % fetal bovine serum) was replaced with D-MEM containing no serum and 10 µM CGS-27023A (Ciba-Geigy), an MMP inhibitor. It is believed that, in the absence of an added MMP inhibitor, MMPs produced by the culture autodigest; thus, addition of

10 an MMP inhibitor to the culture medium resulted in a detectable MMP13 band.

Forty-eight hours after the addition of serum-free medium containing the MMP inhibitor, 10 ml of supernatant were collected and concentrated about 200-fold using Centriprep-30 and Centricon-10 concentrators (Amicon), after which an equal volume of 2X Tris-glycine SDS running buffer was added to each sample. The samples

15 were then applied to a 4-16% pre-stained beta-casein zymogram SDS polyacrylamide gel (Novex). After electrophoresis, the gels were renatured in renaturing buffer (Novex) for 30 minutes at room temperature, followed by overnight incubation at 37°C in zymogram developing buffer (Novex).

Results

20 Normally, MMPs are synthesized as precursors (*i.e.*, procollagenases or zymogens) whose enzymatic activity is latent; proteolytic removal of the proregion after secretion produces the enzymatically active protein. The need for proteolytic processing is circumvented by the use of a procollagenase variant that is enzymatically active even when uncleaved. The constitutively active MMP13 variant used as the transgene contains

25 a proline to valine substitution in the sequence encoding the PRCGVPDV (SEQ ID NO:7) region, which is highly conserved among MMPs and important for controlling enzyme latency.

The activity of the altered MMP13 protein, was determined on a casein zymogram (data not shown). Three MMP species are secreted from control HeLa cells.

30 One runs at the correct molecular weight for the 92 kDa MMP9 enzyme, also referred to as

gelatinase B. Cleavage of the prodomain of MMPs during enzyme activation results in a loss of approximately 10 kDa. The molecular weight of a second band is consistent with the processed form of gelatinase B. Stromelysin-1 (MMP3) and stromelysin-2 (MMP10) are both 54 kDa and one or both may represent the fourth band. Pro-MMP13 is expected to migrate ~60 kDa, which is observed in lane 1 containing uncleaved recombinant MMP13. HeLa cells were transfected with constructs expressing both parental and mutant MMP13. Only the ~60-kDa pro-MMP13 form was detected from cells expressing both constructs, indicating that autoproteolysis of the proenzyme to the mature form is not likely to occur in an exogenous system. These results showed that the proline to valine substitution did not interfere with its native MMP13 activity or substrate specificity.

This method provides a rapid screen for MMP13 variants that retain MMP13 enzymatic activity.

Example 3: Construction of Transgenic Vectors

15 Construction of MMP-13* Linked to tet07

In a further step, cDNA encoding MMP-13* was operably linked to a transcriptional regulatory sequence derived from the tet07 promoter as follows:

The BS(SK-) vector (Stratagene) was digested with KpnI and NotI. A synthetic duplex oligonucleotide having the following sequence was digested with KpnI and Not I and ligated to the vector:

5'-GGTACCACTAGTAAGCTTAGATCTCATATGGTCGACCCCGGGGAATTCCTGC
AGGGATCCTCTAGAAGTACTCCATGGGTATACATCGATGCGGCCGC-3', SEQ ID
NO:10

The BS(SK-) vector as modified above was digested with XbaI and NcoI. A 745 bp fragment containing the SV40 splice site and polyadenylation signal, which was obtained by digesting pcDNA1/Amp (Invitrogen, Carlsbad, CA) with XbaI and NcoI, was ligated to this vector.

The resulting vector was linearized by digestion with XhoI and EcoRI and ligated to a 460 bp XhoI-EcoRI fragment containing the tetO7 promoter region from pUHD 10-3 (Gossen et al., *Proc. Natl. Acad. Sci. USA* **89**:5547, 1992). This vector was

then digested with SpeI, blunt-ended with Klenow polymerase, and digested with EcoRI. pAlter-MMP13* was digested with HindIII, blunt-ended with Klenow polymerase, and digested with EcoRI to obtain an MMP13*-encoding fragment. The MMP13* EcoRI fragment was cloned into the EcoRI digested vector obtained above.

5 The 2792 bp transgene, SEQ ID NO:11 (Figure 2B), was excised by digestion with XhoI and NotI and purified using CsCl gradient centrifugation prior to microinjection into fertilized eggs.

Construction of a Collagen Type II-Promoter-Linked tTa Gene

A gene encoding a tTA repressor-activator fusion protein was operably linked
10 to a joint-specific (type II collagen) promoter.

The modified BS(SK-) vector containing the SV40 splice site and polyadenylation signal as described in Example 1 above was digested with NdeI and Sma I and ligated to a 1897 bp fragment containing the collagen II promoter and enhancer. This fragment was obtained by digesting plasmid PBSΔH1 with HindIII, after which it was
15 blunt-ended with Klenow and digested with NdeI.

The plasmid was then digested with EcoRI and BamHI and ligated to a 1025 bp fragment encoding the tetracycline/VP16 repressor-activator fusion protein that had been excised from the pUHG15-1 plasmid (Gossen et al., *supra*) using EcoRI and BamHI. The plasmid was linearized by digestion with BglII, dephosphorylated using calf intestinal
20 phosphatase, and ligated to a 1554 BamHI enhancer fragment obtained from plasmid PBSΔH1.

Finally, the 5276 bp transgene, SEQ ID NO:12 (Figure 2A), was excised from the vector by digestion with KpnI and NotI, gel purified, purified by CsCl gradient centrifugation, dialyzed against microinjection buffer (5 mM Tris-HCl pH 7.4, 0.1 mM
25 EDTA pH 8.0) and used for microinjection.

Type II collagen promoter-β-galactosidase gene

A reporter gene, suitable for assessing the tissue-specific expression conferred by the type II collagen promoter, was operably linked to the type II collagen promoter.

A 4179 bp BamHI-BglII fragment containing the β-galactosidase gene fused
30 to the β-globin splice sequence and polyadenylation signal was excised from plasmid

pUGH16-3 (Gossen et al., *supra*) and cloned into the BamHI site of unmodified BS(SK-) (Stratagene). This plasmid was digested with EcoRI and HindIII and ligated to a 655 bp Hind III-Eco RI fragment containing the type II collagen promoter sequence, which was excised from the plasmid described above. The plasmid was then digested with EcoRI and
5 ligated to a 2807 bp Eco RI fragment which had been excised from the type II collagen promoter plasmid described above. Restriction mapping was used to verify the orientation of each insert. The 7664 bp transgene, SEQ ID NO:13 (Figure 3A), was excised by digestion with HindIII and NotI, gel purified, purified by CsCl gradient centrifugation, dialyzed against microinjection buffer (5 mM Tris pH 7.4, 0.1 mM EDTA pH 8.0), and
10 used for microinjection into mouse embryos.

Results

Figure 2A and 2B shows a schematic diagram of the synthetic genes generated to achieve regulated expression of MMP13 in chondrocytes. Inducible expression of the transgene was accomplished using the tetracycline regulatable gene
15 expression system (Gossen et al., *supra*; Furth et al., *Proc. Natl. Acad. Sci. USA*, 91:9302-9306, 1994). The first construct shown in Figure 2A places expression of the tetracycline-controlled VP16 transactivator fusion protein under the control of the type II collagen gene promoter. This construct directs expression of the VP16 fusion protein to chondrocytes. The second construct shown in Figure 2B places expression a cDNA
20 encoding a modified version of MMP13 protein (MMP13*) under the direction of the VP16 fusion protein. In the presence of doxycycline, a tetracycline analog provided in the drinking water, the VP16 fusion protein does not bind to the Tet07 promoter of the synthetic MMP13* gene and the gene is silent. Upon removal of doxycycline, the transactivator stimulates transcription of the human MMP13* cDNA and the production of
25 the modified protein product.

In addition to verifying the MMP13* activity, prior to microinjection, both transgene constructs shown in Figure 2A and 2B were tested in primary bovine chondrocytes and in embryonic mouse fibroblast. The results showed the ability of the collagen II promoter to induce expression of a second construct containing either the
30 Tet07-luciferase or the Tet07-MMP13* (data not shown).

Figure 3A is a schematic illustration of a transgene used to assess tissue-specific regulation conferred by type II collagen promoter. The nucleic acid construct comprises: (i) sequences derived from a rat type II collagen promoter; (ii) sequences encoding bacterial β -galactosidase (Lac Z); and (iii) sequences comprising an β -globin splice and polyadenylation signal.

Example 4: Production and Characterization of Transgenic Mice Expressing Tetracycline-Regulated LacZ or MMP-13* in Joint Tissues

The following experiments were performed to produce transgenic mice expressing MMP-13* or a LacZ (β -galactosidase) reporter gene.

Materials and Methods

Preparation and testing of transgene mice. To produce mice expressing MMP-13* under tetracycline regulation, an XhoI-NotI tet07-MMP-13* DNA fragment of about 2784 base pairs (Figure 2B) and a KpnI - NotI CPE-tTA DNA fragment of about 5265 base pairs (Figure 2A) were co-microinjected into fertilized mouse embryos in equimolar amounts. To produce mice expressing the reporter gene, a HindIII-NotI LacZ 7641 base pairs fragment (Figure 3A) was injected into (FVB/N) fertilized eggs as described (Hogan et al., *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratories, 1996). (The size of the restriction fragments is smaller than the transgenes described in Example 3 because the endonuclease cleavage sites are internal to the transgenes.)

Founder animals were first identified by PCR as follows. The tTA-encoding transgene was identified using a primer corresponding to the tTA sequence (5'-CGAGGGCCTGCTCGATCTCC-3', SEQ ID NO:14) and a primer corresponding to a 3' untranslated sequence (5'-GGCATTCCACCACTGCTCCC-3', SEQ ID NO:15). The resulting PCR product was 584 bp in size. The MMP13* -encoding transgene was identified using primers corresponding to sequences encoding MMP13* (5'-GAGCACCTTCTCATGACCTC-3', SEQ ID NO:16) and the 3' untranslated region, respectively. The resulting PCR product was 731 bp in size.

The LacZ-encoding transgene was identified using primers corresponding to

the nuclear localization signal of the β -galactosidase gene (5'-GTTGGTGTAGATGGGCGCATCG-3', SEQ ID NO:17) and the collagen promoter (5'-GCGGGGTCTCAGGTTACAGCC-3', SEQ ID NO:18). The resulting PCR product was 673 bp in size.

5 Southern blot analysis of tail DNA digested with BamHI/NcoI or PvuII/NcoI and hybridized to the 3' untranslated region under high stringency conditions was performed to confirm the results obtained using PCR. The number of copies of transgene DNA that integrated into the genome was determined by comparing the relative intensity of the hybridization signal from transgenic mice with that obtained using control DNAs
10 containing 10 and 100 genome equivalents of the same DNA that was injected.

Copy number was confirmed using Taqman quantitative PCR according to manufacturer's specifications (Perkin Elmer). Transgenic lines were generated by mating founder animals to FVB/N wild-type mice and the subsequent generations were identified by PCR.

15 All mice were administered doxycycline (Sigma Chemical Co., St. Louis MO) prepared as a 100 mg/ml stock solution in 50% ethanol, and diluted to a final concentration of 1.0 mg/ml in acidic drinking water, which was changed on a daily basis (Schultze et al., *Nature Biotech.* **14**:499, 1996).

Whole Embryo Staining for β -Galactosidase (lacZ) Activity. Wild-type
20 females were mated with transgenic males harboring the CPE-lacZ construct. On embryonic day 16 (E16), the females were sacrificed, and the embryos stained for β -galactosidase activity as described (Hogan et al., *supra*, 1996). Prior to fixation, tails from the E16 embryos were removed, digested and analyzed for transgene transmission by PCR.

25 *Expression Analysis via RT-PCR.* Transgene expression was assayed by RT-PCR. Total RNA was isolated from tissues following homogenization in Trizol (Life Technologies). First strand synthesis was generated using the Superscript preamplification kit by Gibco/BRL. Briefly, 5 μ g of total RNA was treated with DNase I for 15 minutes at RT, then inactivate by the addition of 2 μ l of 25 mM EDTA and heated to 65°C for fifteen
30 minutes. Following, the RNA was annealed to 0.5 μ g oligo dT and reverse transcribed

according to manufacturer's specifications. PCR analysis of the cDNA was done using the following primers sets (5' to 3' sequences): tet activator:

CGCCCAGAAGCTAGGTGTAGAG (SEQ ID NO:19) and

CGGCCATATCCAGAGCGCCG (SEQ ID NO:20); MMP13*:

5 GCCCTCTGGCCTGCTGGCTCATG (SEQ ID NO:21) and

CAGGAGAGTCTTGCCTGTATCCTC (SEQ ID NO:22). The resulting PCR products

are 859 bp and 648 bp, respectively. To test the integrity of the mRNA and the efficiency of the reverse transcriptase, each PCR reaction also contained the following c-fos primer set: 5'-AGGAGGGAGCTGACAGATACTCC-3' (SEQ ID NO:23) and

10 5'-AGGCCACAGACATCTCCTCTGG-3' (SEQ ID NO:24). PCR analysis was

performed on the cDNA using Taq-gold (Perkin Elmer) for 10 minutes at 95°C, followed by 35 cycles of 60 seconds at 96°C, 90 seconds at 67°C, and 60 seconds at 72°C. A final twelve minute extension was done at 72°C. Reaction products were run on 2.0% agarose gels and visualized by ethidium bromide staining.

15 ***Embryonic Fibroblast/RT-PCR.*** Tet07-lacZ females were mated to transgenic males harboring both the CPE-tTA and the Tet07-MMP13* transgenes. On E15, the females were sacrificed, and fibroblast prepared from the embryos (Graham et al., *Virology*, 52:456, 1973; Lopata et al., *Nucl. Acids Res.*, 12:5707, 1984). The fibroblast were cultured in DMEM (Gibco/BRL) containing 10% FBS (Gibco/BRL). The fibroblast

20 were subsequently transfected via the calciumphosphate precipitation method with the desired expression plasmids. Forty-eight hours after transfection, total RNA was prepared from the transfected cells using the Trizol method (Gibco/BRL). First strand cDNA synthesis was prepared using the SuperScript preamplification system (Gibco/BRL). MMP13* expression was identified using primers specific for human MMP13

25 (GCCCTCTGGCCTGCTGGCTCATG) (SEQ ID NO:21) and

(CAGGAGAGTCTTGCCTGTATCCTC) (SEQ ID NO:22). The resulting PCR was 648 bp in size.

Immunohistochemistry. Expression of MMP13* in the double transgenic lines was further analyzed by immunohistochemistry, using antibodies specific for MMP-
30 13-derived type II collagen cleavage fragments. For this purpose, joints were fixed in 4%

paraformaldehyde in PBS at neutral pH for 60 minutes at room temperature. They were then rinsed twice in PBS, incubated in 0.1M Tris-HCl, pH 7.4, overnight, and partially decalcified in 0.2M EDTA at neutral pH. The samples were transferred to TOC medium and 6-mm frozen sections were obtained using a Hacker/Bright cryostat. The sections
5 were stained with an antibody that recognizes an epitope present in a degradation product of type II collagen, specifically, in the TC^A degradation product, which is also designated the 3/4 piece. Billingham et al., *J. Clin. Invest.* 99:1534, 1997.

Results

Tetracycline and their analogues are known inhibitors of MMP activity. As a
10 result, we compared the serum levels of DOX when 1 mg/ml was added to the drinking water and the *in vitro* IC₅₀. In a MCA fluorescent assay the IC₅₀ = 59.1 μ M, whereas the serum levels measured 2.64 μ M using a zone of inhibition assay. These data show that the amount of DOX in the serum is 22.4 fold below the level at which 50% of MMP activity could be inhibited. Thus, it is unlikely that there is a significant inhibition due to the
15 DOX.

Figure 3B is a photographic illustration of whole mount staining for β -galactosidase activity of embryonic day 16 transgenic mouse embryos expressing the transgene illustrated in Figure 3A. Blue staining (arrows) is evident in the joints throughout the body of the transgenic animal, while no staining is observed in the
20 non-transgenic, wild-type littermate. Specifically, joints including the ankles, knees, hips, phalanges, wrists, elbows, shoulders, and vertebrae showed expression of the transgene. In addition to the cartilage of the joints, cartilage that has not ossified to the bone at this stage of development, *i.e.*, some of the facial, skull, and rib bones, also stained blue. Figure 3C shows an enlargement of the elbow and paw. These data confirm the
25 expression abilities of the type II collagen promoter, and are useful in determining those tissues (joints) that will express the MMP13* transgene.

The constructs shown in Figure 2A and 2B were co-microinjected into fertilized mouse embryos. Out of 112 newborn mice, 7 transgenic founders harboring both transgenes were identified, however, only four of these transgenic lines were capable
30 of breeding. The transgenes were identified by PCR and verified by Southern blot

analysis using a transgene-specific probe (data not shown). The copy number for each of the four transgenics was further accessed using Taqman quantitative PCR (data not shown). Briefly, transgene copy number ranged from 1-32 and 1-20 for the tet activator and MMP13*, respectively. Specifically, line 6 contained ~8 copies of the tet activator and ~3 copies of the MMP13* transgene.

Expression of the TA and MMP13* transgenes were initially evaluated in the hind-knee joints of four-month old mice (line 6). Amplification of the c-fos endogenous cDNA was used as a control to verify the efficacy of each reaction. Figure 4 shows amplification of an 890 bp fragment resulting from a TA-specific primer set. RT-PCR showed the TA transgene to be expressed in transgenic mice, both on and off DOX, but was not expressed in the non-transgenic controls (lanes 4-5). Constitutive expression of the TA is expected since it is driven by a constitutively active promoter. Moreover, expression of the TA is limited to the joints and was not observed by the RT-PCR in other tissues including brain, heart, liver, kidney, spleen, or skeletal muscle (data not shown).

Figure 4B shows amplification of a 645 bp fragment resulting from an MMP13* specific primer set. Note, the MMP13* primer set is specific for human MMP13 and does not react with its endogenous mouse homologue, collagenase-1. RT-PCR showed that MMP13* was not expressed in the non-transgenic controls (lanes 4-5). Lanes 6-7 show that there is expression of the MMP13* transgene in mice maintained on DOX. Removal of DOX from the drinking water induces a significant amount of expression (lanes 8-9). We estimate a 5-10 fold induction. Furthermore, following gel electrophoresis, PCR fragments were transferred to a nylon membrane and hybridized to a TA or MMP13* specific probe to verify the identity of the PCR product (data not shown).

Fibroblasts from several transgenic lines (such as, e.g., lines 8 and 42) were capable of expressing MMP13*, as evidenced by the appearance of a PCR product of the predicted size. No MMP13* RT-PCR band was detected from cells transfected with vehicle alone. These results indicated that, in these mice, the MMP13* transgene is integrated into a transcriptionally active region of the chromatin.

As early as 3 days after removal of the mice from doxycycline, MMP-13

cleavage products could be detected immunologically. After 30 days without doxycycline, a substantial increase in staining in the growth plate and in the articular cartilage could be seen (Figure 5), but the results differed among different lines of mice (see Table 1 below).

Table 1

Immunohistochemistry				
F1 Animal	Days off DOX	hMMP13 Ab	Type II Collagen Cleavage Fragments Ab	Loss of Safranin O Stain
Line 8	wt	-	-	not remarkable
Line 8	0 d	-	-	" "
	3 d	+	+	" "
	7 d	++	++	Mild
	14 d	+++	+++	Moderate
	wt	-	-	not remarkable
Line 6	30 d			Moderate
Line 8	30 d	+++	+++	Moderate
Line 42	30 d	+	-	not remarkable

Example 5: Phenotype of MMP13* Transgenic Mice

Materials and Methods

To study the effect of MMP13* activity on cartilage in adult transgenic animals, mice were withdrawn from doxycycline for increasing times, after which they were sacrificed. Paraffin-embedded formaldehyde-fixed sections of decalcified cartilage were sectioned and stained with (i) hematoxylin and eosin (H&E) and (ii) Safranin O followed by fast green (American Histo Labs, Gaithersburg MD). Staining techniques in articular cartilage have been described (Peter et al., *J. Exp. Pathol.* **71**:19, 1990).

Results

Control transgenic animals that lack MMP13* expression retain a significant amount of safranin O stain in both the articular cartilage as well as the growth plate of their patella (Figure 6A). By contrast, transgenic animals from line 8 show a substantial loss of safranin O staining in their joints following doxycycline withdrawal. After seven days, a mild reduction of safranin O staining is observed in the articular cartilage of the patella (Figure 6B), which progresses by day 14 to moderate loss of stain in articular

cartilage as well as the growth plate (Figure 6C). A significant loss of safranin O stain was also observed in the other joints including the cartilage of the tarsus and femur, as well as wrist and knuckle, indicating a reduced proteoglycan concentration in these areas compared to controls.

5 To access any changes in the articular cartilage due to transgene expression, mice from line 6 were maintained or removed from DOX for 114 days, and their joints were sectioned and stained with H&E. When compared with an age matched littermate, control the transgenic removed from DOX developed a pathology reminiscent of osteoarthritis. Shown in Figure 7A, the control animal showed no lesions or other
10 osteoarthritis pathologies, whereas the transgenic animal shows the formation of lesions in its articular cartilage (Figure 7B). More specifically, the H&E sections show considerable loss of cartilage, focal erosions, erosions that extend into the bone, and an inflamed synovium. Within the synovium, there is evidence of fibroid necrosis, metaplasia, and synovial cell hyperplasia. In addition to these symptoms of osteoarthritis, some changes
15 observed are more characteristic of rheumatoid arthritis. These changes include angiogenesis as seen by an infiltration of red blood cells, monocytes, and macrophages. Figures 7C and 7D show the synovium at a higher magnification.

Discussion

Transgenic mice expressing a constitutively active human MMP13 protein in
20 the articular cartilage of adult animals have been successfully created. We showed that a unique combination of technologies, *i.e.*, the tetracycline regulatable gene expression system and chondrocyte specific expression of a constitutively active MMP protein, has enabled us to develop a transgenic model resulting in lesion formation and other osteoarthritis pathologies. Using a regulatable/inducible system enabled us to bypass
25 deleterious embryonic effects, while allowing significant hMMP13* expression in the adult mouse. The Lac Z staining shown in Figure 3, demonstrates that type II collagen expression occurs during development at about stage E16 and that type II collagen is involved during the formation of the skeleton. Type II collagen, as well as type I collagen are known to be substrates for proteolytic cleavage by MMP13. Therefore, we predicted
30 that unregulated expression of MMP13 during embryogenesis would be lethal.

Four transgenic lines containing both transgenes were established. In the two lines that expressed hMMP13*, one expressed at very low levels, whereas the other line expressed significant amounts of hMMP13*. The line that expressed only marginal amounts of transgene displayed histological evidence of proteoglycan loss. However, after six months off DOX no lesions were observed. Line 6, which expressed significantly greater amounts of hMMP13*, showed osteoarthritis pathologies including lesion formation, cartilage degradation, and an inflamed synovium after four months off DOX. Moreover, expression of hMMP13* in line 6 can be controlled, *i.e.*, turned on and off by the investigator. This result provides additional evidence that the phenotype is due to expression of hMMP13* and not the result of the integration site in the chromatin.

As observed in line 6, the joint destruction/erosion, lesions, fibroid necrosis, metaplasia, synovial cell hyperplasia and an inflamed synovium (in the absence of T-cells) are among pathologies observed in patients with osteoarthritis. However, not all of the pathologies observed in the transgenics are reminiscent of osteoarthritis. For example, angiogenesis and infiltration of monocytes and macrophages are pathologies observed during the inflammation process associated with rheumatoid arthritis. Note, the absence of neutrophils in the synovial fluid. Migration of neutrophils to the site of inflammation is a hallmark pathology of rheumatoid arthritis.

The data presented in this paper provides direct evidence that MMP13 is a critical player in the development of osteoarthritis. Moreover, the transgenics described in this paper provide an animal model to test the efficacy of therapeutics. Compounds that modulate the activity of MMP13 or inhibit progression of osteoarthritis can be monitored by determining lesion formation and other OA pathologies at various times during the progression of the disease. The ability to turn on and off hMMP13* expression, thus controlling production/timing of lesion formation will be advantageous to determining the compound efficacy.

This OA-like transgenic model can also be used to answer a growing list of biological questions. In addition to MMP13, interstitial collagenase (MMP1) and neutrophil collagenase (MMP8) have been shown to cleave type II collagen. Thus, transgenic mice expressing constitutively active MMP1 or MMP8 would be expected to

show a similar phenotype in their articular cartilage.

Example 6: Augmentation of the Development of Symptoms of Joint Degenerative Disease in MMP-13 Transgenic Mice

5

The following treatments are performed to enhance the symptoms of joint degeneration exhibited by the transgenic animals of the invention.

A group of transgenic mice are treated to induce expression of the transgenes at 4-12 weeks of age. Two to six weeks after induction, the mice are injected
10 intraperitoneally with an inflammatory agent, including without limitation, lipopolysaccharide (10-100 μ g), zymosan (1-10 mg), the superantigen Staphylococcal Enterotoxin B (1-100 μ g), or TGF- β (1-10 μ g). Alternatively, the animals are injected intraarticularly with an inflammatory or chondrocyte function-modulating agent, including without limitation, lipopolysaccharide (1-100 ng), zymosan (50-250 μ g), papain (10-100
15 μ g), TGF- β (0.01-1 μ g), Bone Morphogenic Protein -2 (2-1000 ng), IL-1 (1-100 ng), TGF- α (10-200 ng), IGF (0.01-1 μ g), or FGF (0.01-1 μ g). Age- and sex-matched transgenic mice maintained under a regimen in which the transgenes are not expressed receive the same treatment and serve as controls.

The development of symptoms of degenerative joint disease is monitored by
20 gross observation of joint swelling and function, and by histological evaluation of the joint at selected timepoints after exposure to the inflammatory agent.

The agents will induce an acute inflammatory response and/or transient loss of proteoglycan with a duration of less than one week. The acute inflammatory response and/or transient cartilage changes will upregulate gene expression in the chondrocytes,
25 enhancing the expression of the transgene and increasing the levels of MMP-13 produced.

All patents, applications, articles, publications, and test methods mentioned above are hereby incorporated by reference in their entirety.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed description. Such obvious variations are
30 within the full intended scope of the appended claims.

Claims:

1 1. A transgenic non-human mammal or the progeny thereof having
2 somatic and germline cells which contain, in stably integrated form, a recombinant gene
3 encoding a polypeptide comprising an enzymatically active matrix-degrading enzyme,
4 wherein said recombinant gene is selectively expressed in chondrocytes of said mammal
5 and said expression results in pathological symptoms characteristic of cartilage-
6 degenerative disease.

1 2. A transgenic animal or the progeny thereof having somatic and
2 germline cells which contain a stably integrated first recombinant gene encoding a
3 polypeptide selected from the group consisting of MMP-1, MMP-2, MMP-3, MMP-7,
4 MMP-8, MMP-9, MMP-10, MMP-13, MMP-14, MMP-15, MMP-16, MMP-17 and
5 enzymatically active variants thereof.

1 3. A transgenic animal as defined in claim 2, wherein said first
2 recombinant gene is selectively expressed in synovial chondrocytes of said animal.

1 4. A transgenic animal as defined in claim 2, wherein said first
2 recombinant gene encodes MMP-13.

1 5. A transgenic animal as defined in claim 4, wherein said MMP-13 is
2 human.

1 6. A transgenic animal as defined in claim 4, wherein said first
2 recombinant gene encodes a variant MMP-13 polypeptide comprising enzymatically active
3 proMMP-13.

1 7. A transgenic animal as defined in claim 6, wherein said recombinant
2 gene comprises a MMP-13-encoding sequence as depicted in SEQ ID NO:1.

1 8. A transgenic animal as defined in claim 2, wherein said animal is
2 selected from the group consisting of mouse, rat, rabbit, sheep, cow, goat, and pig.

1 9. A transgenic animal as defined in claim 8, wherein said animal is a
2 mouse.

1 10. A transgenic animal as defined in claim 2, wherein expression of said
2 recombinant gene is under the control of a first regulatable promoter.

1 11. A transgenic animal as defined in claim 10, wherein said first
2 regulatable promoter comprises tetO7.

1 12. A transgenic animal as defined in claim 11, wherein said promoter has
2 the sequence depicted in SEQ ID NO:2.

1 13. A transgenic animal as defined in claim 10, further comprising a
2 second recombinant gene encoding a polypeptide that regulates said first promoter.

1 14. A transgenic animal as defined in claim 12, wherein expression of said
2 second recombinant gene is under the control of a second regulatable promoter.

1 15. A transgenic animal as defined in claim 13, wherein said second
2 regulatable promoter comprises sequences derived from a type II collagen promoter that
3 confer selective expression of said second recombinant gene in joint tissues.

1 16. A transgenic mouse or the progeny thereof having somatic and
2 germline cells which contain in stably integrated form:

3 (i) a first recombinant gene comprising a sequence encoding a
4 variant MMP-13 polypeptide comprising MMP-13*, wherein said sequence is operably
5 linked to a tetO7 promoter, and

6 (ii) a second recombinant gene encoding a tTA protein operatively
7 linked to sequences derived from a type II collagen promoter.

1 17. A transgenic mouse as defined in claim 16 wherein expression of said
2 recombinant genes in joint tissue results in pathological symptoms characteristic of joint
3 degenerative disease.

1 18. An isolated nucleic acid encoding enzymatically active proMMP-13,
2 wherein said nucleic acid has a sequence selected from the group consisting of the
3 sequence depicted in SEQ ID NO:1, sequence-conservative mutants thereof, and function-
4 conservative mutants thereof.

1 19. A recombinant cloning vector comprising a nucleic acid as defined in
2 claim 18.

1 20. A host cell comprising a vector as defined in claim 19.

1 21. A method for producing a polypeptide comprising culturing a cell as
2 defined in claim 20 under conditions appropriate for expression of said enzymatically
3 active proMMP-13.

1 22. A method for producing phenotypic changes associated with cartilage-
2 degenerative disease in a mammal, comprising maintaining a mammal as defined in claim
3 1 under conditions in which said recombinant gene is selectively expressed in joint tissue
4 of said mammal.

1 23. A method for producing phenotypic changes associated with cartilage-
2 degenerative disease in a mammal, comprising maintaining a mammal as defined in claim
3 2 under conditions in which said recombinant gene is selectively expressed in joint tissue
4 of said mammal.

1 24. A method for producing phenotypic changes associated with cartilage-
2 degenerative disease in a mouse, comprising maintaining a mouse as defined in claim 16
3 for a predetermined time in the absence of tetracycline or biologically active analogues
4 thereof.

1 25. A method for determining the potential of a composition to counteract
2 cartilage-degenerative disease, said method comprising:

3 (i) administering a known dose of the composition to a transgenic
4 animal as defined in claim 1 under conditions in which phenotypic indicators associated
5 with cartilage-degenerative disease are expressed;

6 (ii) monitoring development of the phenotypic indicators of
7 cartilage-degenerative disease for a predetermined time following administration of the
8 composition; and

9 (iii) comparing the extent of the phenotypic indicators in the animal
10 to which the composition was administered relative to a control transgenic animal that had
11 not been exposed to the composition,

12 wherein any difference in the nature or extent of the phenotypic indicators, or any
13 difference in the time required for the phenotypic indicators to develop, indicates the
14 potential of the composition to counteract cartilage-degenerative disease.

1 26. A method for determining the potential of a composition to counteract
2 cartilage-degenerative disease, said method comprising:

3 (i) maintaining a transgenic animal as defined in claim 16 for a
4 predetermined time in the absence of tetracycline or a tetracycline analogue, wherein said
5 maintenance results in the appearance of one or more phenotypic indicators of cartilage-
6 degenerative disease in said animal;

7 (ii) administering a known dose of said composition to the animal;

8 (iii) monitoring development of said phenotypic indicators for a
9 predetermined time following administration of the composition; and

10 (iii) comparing the extent of the phenotypic indicators in the animal to
11 which the composition was administered relative to a control transgenic animal that had
12 not been exposed to the composition,
13 wherein any difference in the nature or extent of the phenotypic indicators indicates the
14 potential of the composition to counteract cartilage-degenerative disease.

1 27. A method for determining the potential of a composition to counteract
2 cartilage-degenerative disease, said method comprising:

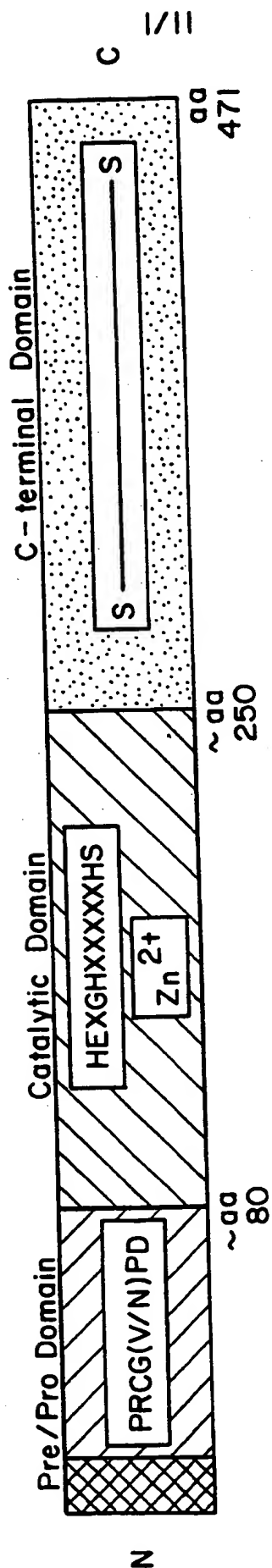
3 (i) providing a transgenic animal as defined in claim 16 that had
4 been maintained in the presence of tetracycline or a tetracycline analogue to repress
5 expression of said first recombinant gene;

6 (ii) substantially simultaneously (a) administering to said animal a
7 known dose of said composition and (b) withdrawing said tetracycline;

8 (iii) monitoring development of phenotypic indicators of cartilage-
9 degenerative disease for a predetermined time following administration of the
10 composition; and

11 (iv) comparing the extent of the phenotypic indicators in the animal
12 to which the composition was administered relative to a control transgenic animal that had
13 not been exposed to the composition,
14 wherein any difference in the nature or extent of the indicators, or any difference in the
15 time required for the indicators to develop, indicates the potential of the composition to
16 counteract cartilage-degenerative disease.

FIG. 1A



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FIG. 1B

CAAGATGCATCCAGGGTCCCTGGCTGCCTTCCCTCTTCTTGTGAGCTGGACTCATTTGTCGGGCCCTTCCCTTCCAGTGGTGGTGAAGA
 M H P G V L A A F L F L S W T H C R A L P L P S G G D E D
 TGATTGTCTGAGGAAGACCTCCAGTTTGACAGCGCTACCTGAGATCATACTACCATCTTACAAATCTCGGGGAATCCTGAAGGAGAA
 D L S E E D L Q F A E R Y L R S Y Y H P T N L A G I L K E N
 TGCAGCAAGCTCCATGACTGAGAGGCTCCGAGAAATGCAGTCTTCTTCGGCTTAGAGGTGACTGGCAAACTTGACGATAACACCTTAGA
 A A S S M T E R L R E M Q S F F G L E V T G K L D D N T L D
 TGTCTGAATAAGCCCAAGATCGGGGTTGTTGATGTGGTGAATACAATGTTTCCCTCGAACTCTTAAATGGTCCAAATGAATTAAAC
 V M K K P R C G V V D V G E Y N V F P R T L K W S K M N L T
 CTACAGAATTGTGAATTACACCCCTGATATGACTCATTTCTGAAGTCGAAAGGCATTCAAAAAGCCTTCAAAGTTTGTCCGATGTAAC
 Y R I V N Y T P D M T H S E V E K A F K A F K V W S D V T
 TCCTCTGAATTTACAGACTTCACGATGGCATTTGCTGACATCATGATCTCTTTTGGAAATTAAGGAGCATGGCGACTTCTACCCATTGA
 P L N F T R L H D G I A D I M I S F G I K E H G D F Y P F D
 TGGGCCCTCTGGCCCTCATGCTTTTCCCTCCTGGGCCAAATTAAGGAGAGATGCCCATTTTGATGATGATGATAAACCTGGACAAG
 G P S G L L A H A F P P G P N Y G G D A H F D D D E T W T S
 TAGTTCCAAAGGCTACAACCTTGTCTTGTGCTGGCATGAGTTTCGGCCACTCCTTAGGTCTTGACCACTCCAAGGACCCCTGGAGCACT
 S S K G Y N L F L V A A H E F G H S L G L D H S K D P G A L
 CATGTTTCTATCTACACCTACACCGGCAAAAGCCACTTTATGCTTCTGATGACGATGTACAAGGATCCAGTCTCTCTATGGTCCAGG
 M F P I Y T Y T G K S H F M L P D D D V O G I O S L Y G P G

A

A

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FIG. 1C

A

AGATGAAGACCCCAACCTAAACATCCAAAAAGCCAGACAAATGTGACCCCTTCCTTATCCCTTGATGCCATTACCAAGTCTCCGAGGAGA
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 AACAAATGATCTTTAAAGACAGATTCTTCTGGCGCCTGCATCCTCAGCAGGTTGATGCGGAGCTGTTTTTAACGAAATCATTTTGGCCAGA
 T M I F K D R F F W R L H P Q Q V D A E L F L T K S F W P E
 ACTTCCCAACCGTATTGATGCTGCATATGAGCACCCCTTCTCATGACCTCATCTTCATCTTCAGAGGTAGAAAATTTTGGGCTCTTAATGG
 L P N R I D A A Y E H P S H D L I F I F R G R K F W A L N G
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 Y D I L E G Y P K K I S E L G L P K E V K K I S A A V H F E
 GGATACAGGCAAGACTCTCCTGTTCTCAGGAAACCAAGGCTGGAGATATGATGATACATAACCATATATTATGGATAAAGACTATCCGAGACT
 D T G K T L L F S G N Q V W R Y D D T N H I M D K D Y P R L
 AATAGAAGAAGACTTCCCAGGAATTGGTGATAAAGTAGATGCTGTCTATGAGAAAAATGGTTATATCTATTTTCAACGGACCCATACA
 I E E D F P G I G D K V D A V Y E K N G Y I Y F F N G P I Q
 GTTTGAATACAGCATCTGGAGTAACCGTATTGTTCCGCTCATGCCAGCAATTCCTATTTTGTGGTGTAAAGTGTCTTTTAAAAAATGTT
 F E Y S I W S N R I V R V M P A N S I L W C *
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FIG. 2A

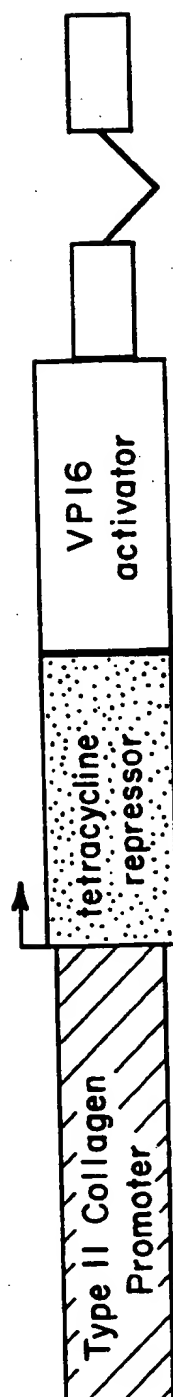
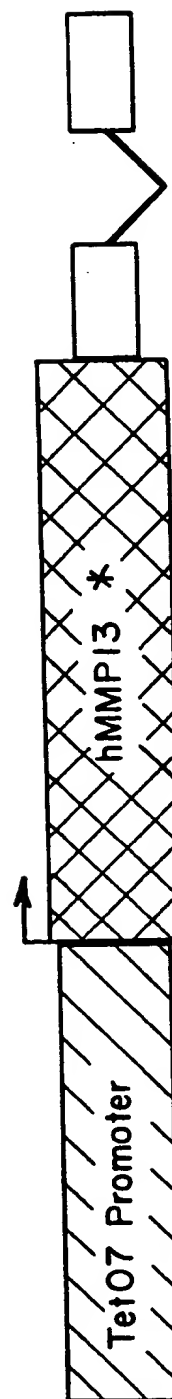
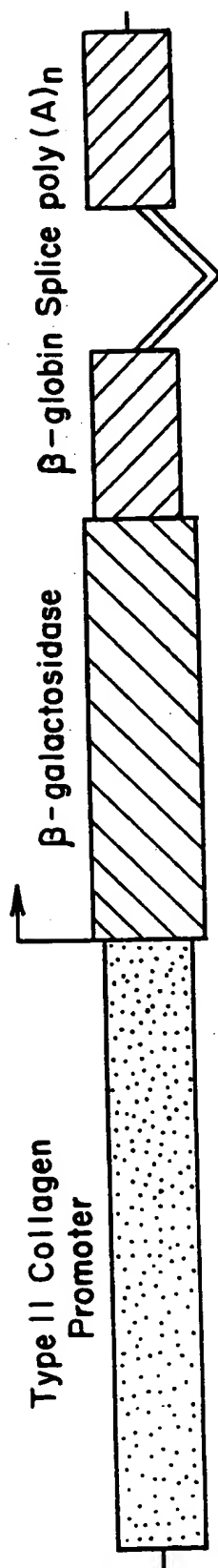


FIG. 2B



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FIG. 3A

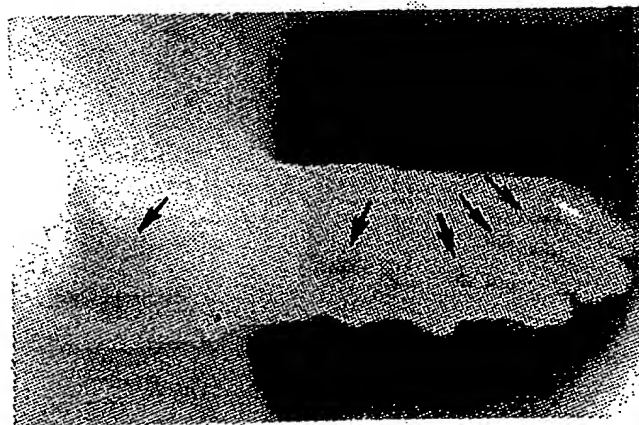


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FIG. 3B



FIG. 3C



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FIG. 4A

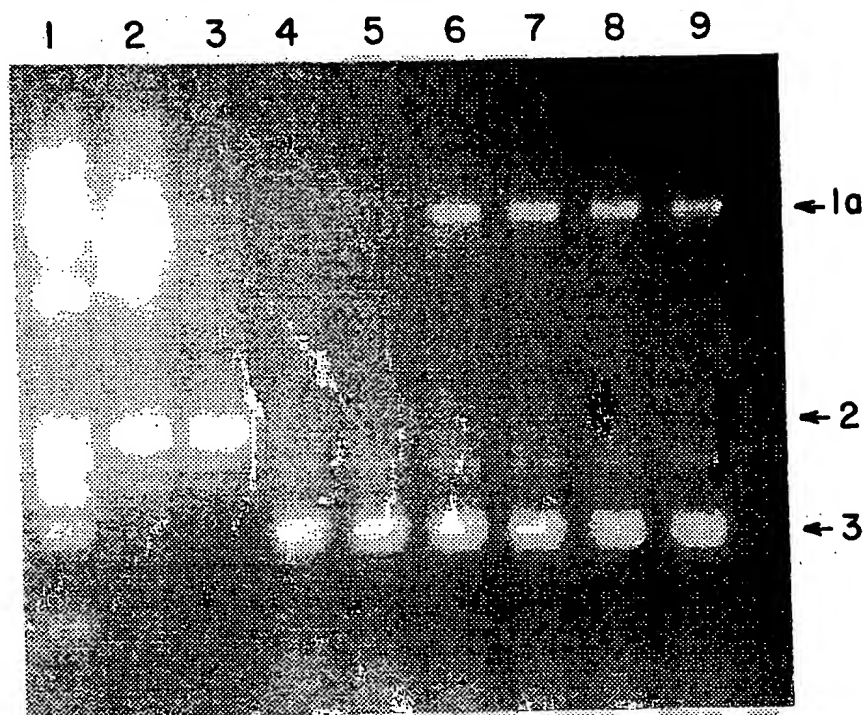
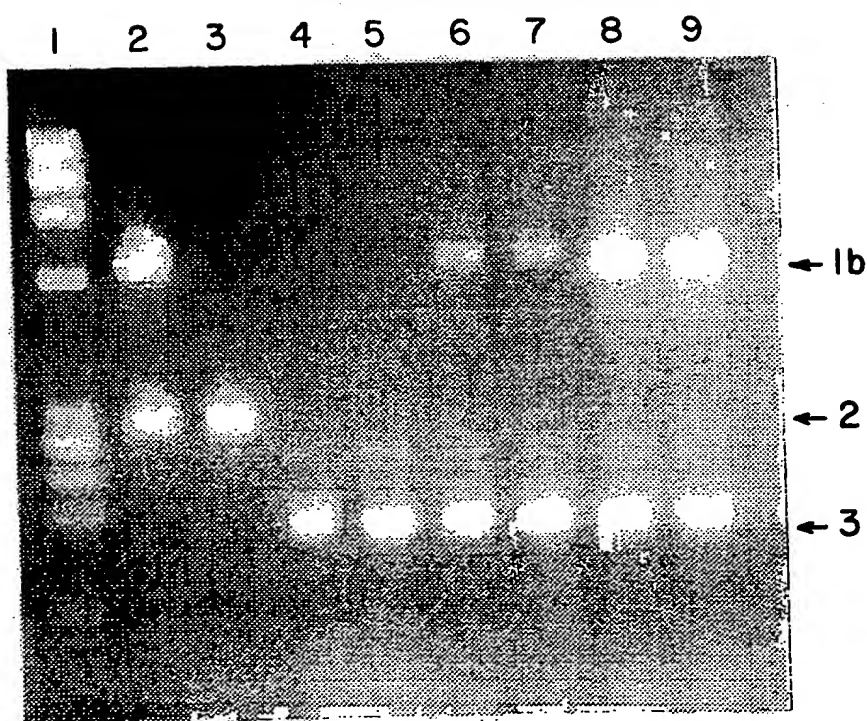


FIG. 4B



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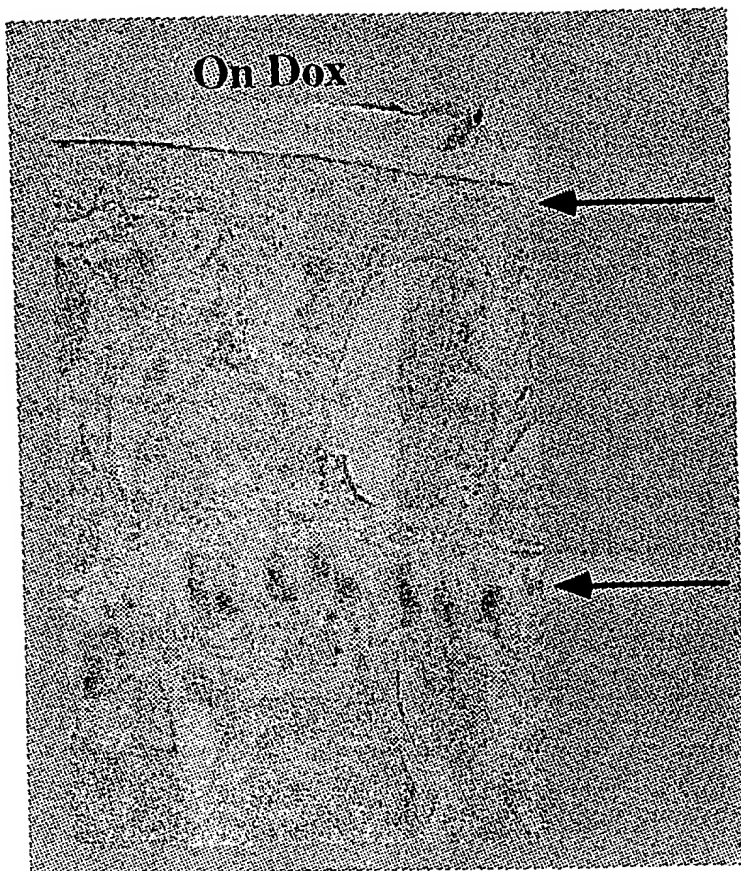


FIG. 5A

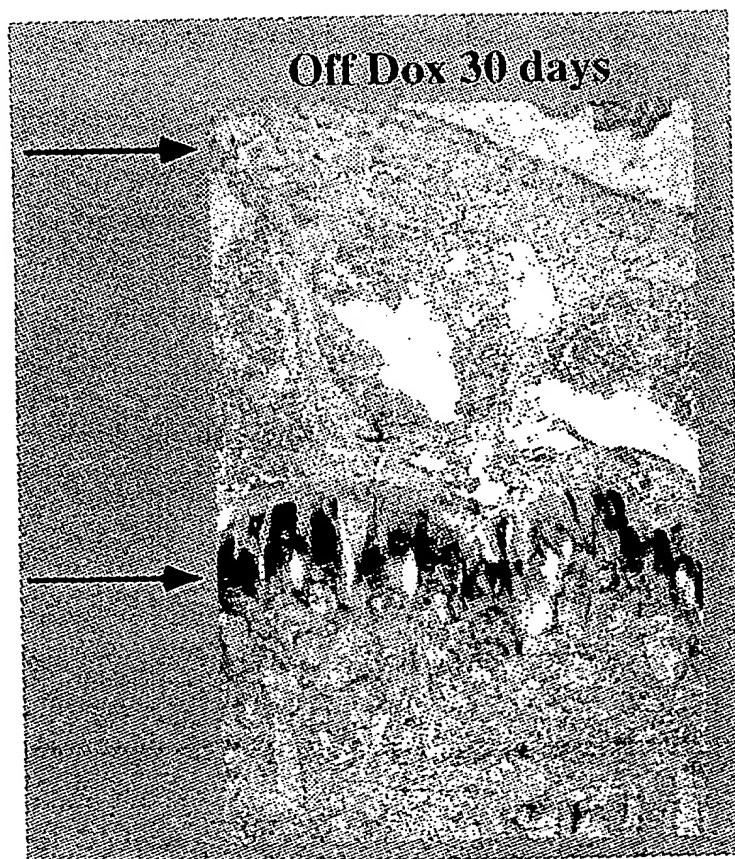
Articular Cartilage

Growth Plate

FIG. 5B

Articular Cartilage

Growth Plate



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FIG. 6C



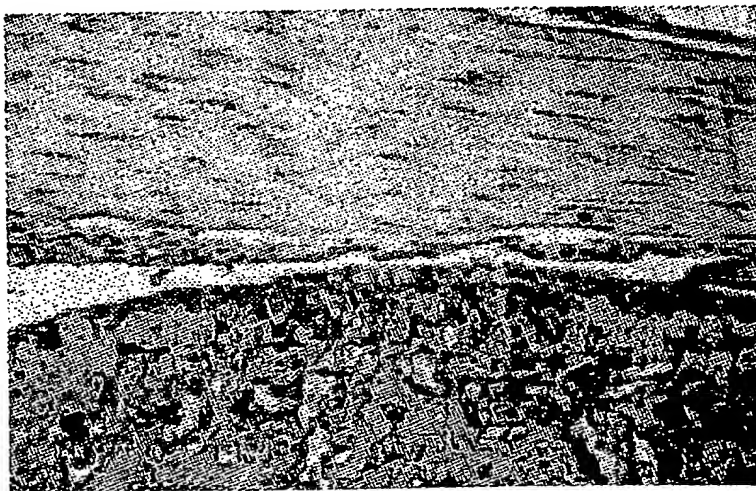
off Dox 14 Days

FIG. 6B



off Dox 7 Days

FIG. 6A



on Dox

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FIG. 7A

FIG. 7B



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FIG. 7C



FIG. 7D

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Killar, Loran

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DISEASES OF CARTILAGE

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 Pro Thr Asn Leu Ala Gly Ile Leu Lys Glu Asn Ala Ala Ser Ser Met
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 Gly Lys Leu Asp Asp Asn Thr Leu Asp Val Met Lys Lys Pro Arg Cys
 85 90 95
 Gly Val Val Asp Val Gly Glu Tyr Asn Val Phe Pro Arg Thr Leu Lys
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 Met Thr His Ser Glu Val Glu Lys Ala Phe Lys Lys Ala Phe Lys Val
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 Trp Ser Asp Val Thr Pro Leu Asn Phe Thr Arg Leu His Asp Gly Ile
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 Ala Asp Ile Met Ile Ser Phe Gly Ile Lys Glu His Gly Asp Phe Tyr
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 Pro Phe Asp Gly Pro Ser Gly Leu Leu Ala His Ala Phe Pro Pro Gly
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 Pro Asn Tyr Gly Gly Asp Ala His Phe Asp Asp Asp Glu Thr Trp Thr
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 Gln Val Trp Arg Tyr Asp Asp Thr Asn His Ile Met Asp Lys Asp Tyr
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<210> 12

<211> 5276

<212> DNA

<213> Artificial Sequence

<220>

<223> tetracycline/VP16 repressor-activator fusion
linked to a type II collagen promoter and
enhancer, "CPE-tTA transgene"

<400> 12

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5276

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<211> 7664

<212> DNA

<213> Artificial Sequence

<220>

<223> beta-galactosidase (lacZ) gene linked to
beta-globin splice sequence and polyadenylation
signal, and linked to type II collagen promoter
sequence

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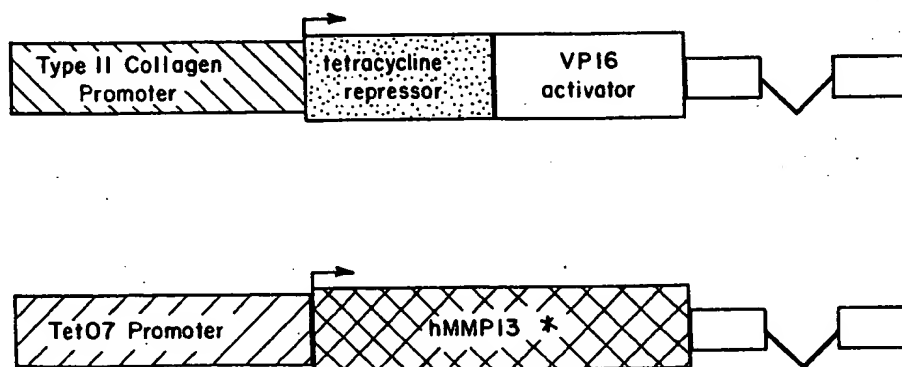
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(74) Agents: BARNHARD, Elizabeth, M.; Patent Law Dept., American Home Products Corporation, One Campus Drive, Parsippany, NJ 07054 (US) et al.		(88) Date of publication of the international search report: 25 November 1999 (25.11.99)	

(54) Title: **TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE**

(57) Abstract

The present invention provides animal model systems for cartilage-degenerative disease, which comprise transgenic animals which can express recombinant matrix-degrading enzymes (MDEs), particularly matrix metalloproteinases (MMPs), in a temporally and spatially regulated manner. The invention also provides methods for producing phenotypic indicators of cartilage-degenerative disease in a mammal and methods for determining the potential of a composition to counteract cartilage-degenerative disease. The invention also provides isolated nucleic acids encoding proMMP polypeptides that exhibit constitutive enzymatic activity and isolated proMMP polypeptides.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/27056

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/57 C12N9/64 C12N5/10 A01K67/027 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D'ARMIENTO J ET AL: "Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema" CELL, vol. 71, 11 December 1992 (1992-12-11), pages 955-961, XP002105587 cited in the application see discussion section ---	2
Y	SHAPIRO SD: "Mighty mice: transgenic technology "knocks out" questions of matrix metalloproteinase function" MATRIX BIOLOGY, vol. 15, no. 8-9, March 1997 (1997-03), pages 527-533, XP002105138 cited in the application page 530, column 2, line 42 - line 45 --- -/-	1-5, 8-10, 13-15, 22,23,25



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

16 September 1999

Date of mailing of the international search report

05. 10. 99

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Lonnoy, 0

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/27056

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEIR E ET AL: "Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 93, September 1996 (1996-09), pages 10240-10245, XP002105139 figure 1A ---	1-5, 8-10, 13-15, 22,23,25
Y	FREIJE J ET AL: "Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 269, no. 24, 17 June 1994 (1994-06-17), pages 16766-16773, XP002105588 figure 1 ---	18-21
Y	SANCHEZ-LOPEZ R ET AL: "Structure-function relationships in the collagenase family member Transin" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 263, no. 24, 25 August 1988 (1988-08-25), pages 11892-11899, XP002105589 see P93->V mutant in section "Self-activating Mutations" on page 11896 ---	18-21
A	KNÄUPER V ET AL: "Biochemical characterization of human collagenase-3" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 271, no. 3, 19 January 1996 (1996-01-19), pages 1544-1550, XP002105702 cited in the application ---	
A	KOZACI L ET AL: "Degradation of type II collagen, but not proteoglycan, correlates with matrix metalloproteinase activity in cartilage explant cultures" ARTHRITIS RHEUM, vol. 40, no. 1, January 1997 (1997-01), pages 164-174, XP002106784 ---	
A	DE 195 01 032 A (MAX DELBRUECK CT FUER MOLEKULA) 18 July 1996 (1996-07-18) ---	
	-/--	

INTERNATIONAL SEARCH REPORT

National Application No

PCT/US 98/27056

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SYMPSON C ET AL: "Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression" J CELL BIOL., vol. 125, no. 3, May 1994 (1994-05), pages 681-693, XP002115516 ---</p>	
A	<p>KNÄUPER V ET AL: "The role of the C-terminal domain of human collagenase-3 (MMP-13) in the activation of procollagenase-3, substrate specificity, and tissue inhibitor of metalloproteinase interaction" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 272, no. 12, 21 March 1997 (1997-03-21), pages 7608-7616, XP002115522 -----</p>	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/27056

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-17, 22-27

A transgenic non-human mammal having somatic or germline cells which contain, in stably integrated form, a recombinant gene encoding an enzymatically active matrix-degrading enzyme and wherein said recombinant gene is selectively expressed in chondrocytes; said transgenic animal wherein said recombinant gene encodes a matrix metalloproteinase (MMP); said transgenic animal wherein said recombinant gene is selectively expressed in synovial chondrocytes; said transgenic animal wherein said recombinant gene encodes human MMP13; said transgenic animal wherein said recombinant gene encodes an enzymatically active pro-MMP13 polypeptide; said transgenic animal wherein expression of said recombinant gene is regulatable; said transgenic animal wherein said regulation of expression of said transgene involves the tetracycline regulation system; a method for producing phenotypic changes associated with cartilage degenerative disease in a mammal involving said transgenic non-human animal; a method for determining the potential of a composition to counteract cartilage-degenerative disease involving said transgenic animal.

2. Claims: 18-21

An isolated nucleic acid encoding enzymatically active pro-MMP13 polypeptide; a recombinant vector comprising said nucleic acid, host cell comprising said vector, and method for producing said polypeptide.

Information on patent family members

PCT/US 98/27056

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